

1977

Isolation and properties of the catalytic subunit of phosphorylase b kinase

James Robert Skuster
Iowa State University

Follow this and additional works at: <https://lib.dr.iastate.edu/rtd>

 Part of the [Biochemistry Commons](#)

Recommended Citation

Skuster, James Robert, "Isolation and properties of the catalytic subunit of phosphorylase b kinase " (1977). *Retrospective Theses and Dissertations*. 6043.
<https://lib.dr.iastate.edu/rtd/6043>

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.
2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.
3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.
4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.
5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

University Microfilms International

300 North Zeeb Road
Ann Arbor, Michigan 48106 USA
St. John's Road, Tyler's Green
High Wycombe, Bucks, England HP10 8HR

77-29,869

SKUSTER, James Robert, 1943-
ISOLATION AND PROPERTIES OF THE CATALYTIC
SUBUNIT OF PHOSPHORYLASE b KINASE.

Iowa State University, Ph.D., 1977
Chemistry, biological

Xerox University Microfilms, Ann Arbor, Michigan 48106

Isolation and properties of the catalytic
subunit of phosphorylase b kinase

by

James Robert Skuster

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1977

TABLE OF CONTENTS

	Page
ABBREVIATIONS	iii
INTRODUCTION	1
MATERIALS AND METHODS	17
RESULTS	21
DISCUSSION	57
LITERATURE CITED	72
ACKNOWLEDGMENTS	75

ABBREVIATIONS

AAT	Aspartate transaminase
ATP	Adenosine triphosphate
cyclic-AMP	3'-5'-Cyclic adenosine monophosphate
EDTA	Ethylenediaminetetraacetate
EGTA	Ethyleneglycol-bis(β -aminoethyl ether)-N,N'-tetraacetate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
LDH	Lactate dehydrogenase
MES	2(N-Morpholino)ethane sulfonic acid
MDH	Malic dehydrogenase
SDS	Sodium dodecyl sulfate
Tetradecapeptide	Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu
S	One Svedberg

INTRODUCTION

Phosphorylase b kinase (ATP-phosphorylase phosphotransferase EC 2.7.1.38) is a key enzyme involved in the regulation of glycogenolysis in skeletal muscle. The enzyme has been purified to electrophoretic homogeneity and its physiochemical, kinetic and regulatory properties have been extensively studied. The activity of phosphorylase kinase can be modulated by several different mechanisms and a scheme has been proposed which links the in vivo regulation of glycogenolysis to the activity of phosphorylase kinase on its substrate glycogen phosphorylase. Phosphorylase catalyzes the degradation of glycogen and exists in two interconvertible forms (1). One form, phosphorylase b, is found in resting muscle and thought to be a physiologically inactive species. Phosphorylase a, the physiologically active species, catalyzes the production of glucose-1-phosphate from glycogen in response to cellular energy demand. Phosphorylase kinase converts phosphorylase b to phosphorylase a by transferring the γ phosphoryl group of ATP to a seryl residue located near the N-terminus of phosphorylase (2). Phosphorylase kinase has an absolute calcium requirement for catalytic activity and the conversion is inhibited by the calcium chelating reagent EGTA as well as preparations of sarcoplasmic reticulum (3-6). Nature has coupled energy production from glycogen and contraction in mammalian muscle by requiring that intracellular free calcium be available for the systems to express their activities.

Phosphorylase kinase is found in and isolated from resting muscle in a form referred to as nonactivated or inactive phosphorylase kinase.

Nonactivated kinase is essentially inactive at physiological pH but nearly fully active at pH 8.2 (7). Inactive kinase can be converted to activated forms by two different phosphorylation mechanisms (8). Phosphorylation increases the activity of the enzyme when measured at pH 8.2 or pH 6.8 but the increase is greater at pH 6.8. One mechanism, thought to be of physiological significance, involves the conversion of nonactivated to activated phosphorylase kinase through phosphorylation by cyclic-AMP dependent protein kinase. The other method of activation by phosphorylation is by auto- or self-phosphorylation of phosphorylase kinase and this mechanism of activation is of questionable physiological significance. Another method of activating phosphorylase kinase is by limited proteolytic degradation of the enzyme. A trypsinized enzyme form has been studied in detail and it has many properties similar to those found for phosphorylase kinase activated by phosphorylation. A more complete discussion of these seemingly unrelated methods of increasing the activity of phosphorylase kinase and its physiochemical and kinetic properties will be presented in the following sections.

Physiochemical properties

Rabbit muscle phosphorylase kinase has been purified to homogeneity and found to have a sedimentation coefficient of 23S to 26.1S (8-10). The molecular weight for the holoenzyme was determined by Cohen (9) and Hayakawa, et al. (10, 11) to be 1.3×10^6 using sedimentation equilibrium. These workers also determined the subunit composition and arrived at significantly different values. Using acrylamide gel electrophoresis in the presence of SDS, Cohen found that the holoenzyme is composed of

four different polypeptides and labeled them α , α' , β , and γ . The molecular weights were reported as $\alpha = 145,000$, $\alpha' = 140,000$, $\beta = 128,000$ and $\gamma = 45,000$. The α' subunit was present in trace amounts and later established to be an isozyme of the α subunit (12). The three major subunits, α , β , and γ were found to exist in equimolar quantities by densitometric analysis of acrylamide gels, by gel filtration in the presence of SDS which separated the γ subunit from an α plus β subunit mixture, and by the distribution of ^{14}C between the subunits after carboxymethylation with iodo $\left[^{14}\text{C}\right]$ acetate suggesting a minimal molecular weight of 318,000 for $(\alpha\beta\gamma)$. The holoenzyme was determined to be a dodecamer with a subunit composition of $(\alpha\beta\gamma)_4$. Hayakawa, et al., using acrylamide gel electrophoresis in the presence of SDS, found the molecular weights of the subunits to be A = 118,000, B = 108,000 and C = 41,000 with a stoichiometry of $A_4B_4C_8$ for the native phosphorylase kinase. The amino acid composition as reported by both workers was not unusual and no disulfides were found. Hayakawa determined the isoelectric point of phosphorylase kinase to be 5.77 using sucrose density gradient isoelectric focusing. The enzyme focused as a single turbid band with several minor bands and no enzymic activity was detected in any of the fractions.

Effect of pH on phosphorylase kinase activity

Phosphorylase b kinase as extracted from resting muscle is present in a form essentially inactive when assayed at pH 6.8 and partially active when assayed at higher pHs. The ratio of activity at pH 6.8 to that at pH 8.2 is less than 0.05 for nonactivated phosphorylase kinase

prepared in crude extract or in highly purified form. The pH 6.8 activity can be increased 25 to 60 fold or more by a variety of treatments and the pH 6.8/8.2 activity ratio is used as a measure of the degree of activation of the enzyme.

During the activity assay of nonactivated phosphorylase kinase at pH 6.8, there is a pronounced lag in the rate of product formation (8), i.e., the enzyme becomes more active during the course of the reaction. The lag is diminished by assaying at a higher pH, preincubation with phosphorylase (13) or by activating the enzyme. The mechanism causing the lag is not understood but it has been suggested that the lag is the result of autophosphorylation (14) or dissociation (13) of phosphorylase kinase during the reaction.

Activation by phosphorylation

The conversion of nonactivated phosphorylase kinase to an activated or phosphorylated form can be catalyzed by phosphorylase kinase itself (referred to as autoactivation or autophosphorylation) and by cyclic-AMP dependent protein kinase. Activation by phosphorylation has been extensively studied for both systems and the properties of the activated enzyme forms described. Nonactivated phosphorylase kinase is auto-activated by incubation of the enzyme with millimolar concentrations of Mg^{2+} and ATP. The characteristics of the reaction resemble that found for the phosphorylase conversion reaction by nonactivated phosphorylase kinase in that; the progress curve of the reaction exhibits a pronounced lag, the reaction is strongly inhibited by EGTA and the rate of the autoactivation reaction is dependent on pH. The reaction

is affected by buffer (most rapid in MES), substrates and various phosphate compounds (14, 15). The autocatalytic reaction involves phosphorylation of the α and β subunits and at least 32 moles of phosphate can be incorporated per mole of holoenzyme. An increase in kinase activity at pH 6.8 parallels total phosphate incorporation (15). The pH 6.8/8.2 activity ratio can be increased to approximately 0.5 with a doubling of the pH 8.2 activity. Carlson and Graves (14) suggested the lag associated with inactive kinase is caused by autophosphorylation during the phosphorylase conversion reaction and many investigators have suggested autophosphorylation may be of physiological significance although experimental evidence is lacking.

Activation of phosphorylase kinase by cyclic-AMP dependent protein kinase has been studied in vivo and in vitro. It is postulated to be a physiological response to hormonal stimulation by epinephrine which acts through the second messenger, cyclic-AMP. In vitro activation by protein kinase is accompanied by rapid phosphorylation of a specific seryl residue in the β subunit. After a short lag period, a second site in the α subunit is phosphorylated more slowly and is not associated with any further increase in activity (9, 11). The stoichiometry of incorporation is nearly one mole of phosphate into each of the two subunits. The pH 6.8/8.2 activity ratio increases to 0.38 with 1.5 to 2 fold increase in the pH 8.2 activity. Phosphopeptides from the sites phosphorylated during the activation reaction have been isolated and their amino acid sequences determined (16,17). The same phosphopeptides were isolated from phosphorylase kinase that had

been activated in vivo by injection of epinephrine into live rabbits providing direct evidence that activation/phosphorylation of phosphorylase kinase is a physiological response to hormonal stimulation (17).

Cohen and Antoniow (18) proposed a mechanism for the regulation of phosphorylase kinase by phosphorylation-dephosphorylation of these specific sites. They suggested that activation of phosphorylase kinase by protein kinase is the result of phosphorylation of the β subunit. Phosphorylation of the α subunit allows for the accelerated dephosphorylation of the β subunit by a phosphorylase kinase phosphatase resulting in the inactivation of the enzyme. Cohen and Antoniow provided support for their hypothesis by showing in vitro that there was a direct correlation between the state of phosphorylation of the β subunit and the level of activation of the enzyme during the phosphorylation-dephosphorylation sequence. Phosphorylation of the α subunit was found to stimulate 100 fold the rate of dephosphorylation of the β subunit by an endogeneous phosphatase yielding preparations of phosphorylase kinase with basal or nonactivated kinase activity, phosphorylated specifically in the α subunit. The role of cyclic-AMP dependent protein kinase phosphorylation of phosphorylase kinase has become less clear with a recent report showing that more than two discrete sites (8 per holoenzyme) can be phosphorylated by protein kinase. In a reaction mixture containing high Mg^{2+} concentrations (10mM), more than 32 moles of phosphate can be incorporated into phosphorylase kinase (15). The authors suggested that activation by

autoactivation and protein kinase activation may involve phosphorylation of common sites.

Activation by proteolysis

Several proteases have been found which activate phosphorylase kinase irreversibly. Trypsin activation of kinase is characterized by a sharp increase in activity at pH 6.8 and a modest increase at pH 8.2 with the pH 6.8/8.2 activity ratio approaching unity (8). The effect of trypsin activation on the subunit structure of kinase has been examined using acrylamide gel electrophoresis in the presence of SDS (9, 11). When phosphorylase kinase is incubated with limiting amounts of trypsin, the increase in the pH 6.8 activity correlates with the disappearance of the α subunit and after an initial increase the pH 8.2 activity remains stable even after the β subunit has nearly disappeared as a result of proteolysis. Degradation of the α and β subunits results in new protein staining bands of lower molecular weights appearing on the gels. The γ subunit is resistant to proteolytic attack and its appearance on gels remains unchanged after the trypsin reaction. During the course of the reaction the pH 6.8/8.2 activity ratio increases to 0.7 and the pH 8.2 activity is lost by more extensive trypsin attacks. The sedimentation coefficient of trypsin activated phosphorylase kinase is 22S (19) indicating that the tryptic fragments are to a large degree still bound to the enzyme core. The proteases KAF (kinase activating factor) and chymotrypsin were reported to activate phosphorylase kinase as effectively as trypsin (20) but trypsin activated kinase has been the most extensively studied proteolytic form.

Calcium and phosphorylase kinase

The role of calcium in the phosphorylase kinase reaction has a long and confusing history. The initial observation that calcium may be important for the phosphorylase conversion reaction was made by Fisher and Krebs when they observed the conversion of phosphorylase in the presence of ATP after crude muscle extracts had been passed through unwashed filter paper (21). It was established that the effect was due to extraction of metals (probably calcium) from the filter paper. Progress on defining the role of calcium in the kinase reaction was hampered by the presence of the calcium dependent protease, KAF, in the early kinase preparations. After KAF had been identified as a protease contaminant, procedures for preparing KAF free phosphorylase kinase were established enabling more satisfactory studies on the role of calcium to be done. Meyer, et al. (3) did make the correct suggestion that phosphorylase kinase has an absolute requirement of calcium for activity even though KAF contaminated their enzyme preparations. EGTA and to a lesser extent, EDTA, were found to inhibit phosphorylase kinase activity and the most effective metal which relieved this inhibition was calcium. They also made the suggestion that calcium may couple muscle contraction and glycogenolysis. Ozawa, et al. (5) and Ozawa and Ebashi (22) presented supporting evidence by showing that the level of free calcium required for half maximal activity of phosphorylase kinase is in the physiological range found for contracting muscle.

Brostrom, et al. (6) reported the K_m values and the dissociation constants of calcium for both nonactivated and activated phosphorylase

kinase. The reported K_m s were 0.2 μ M at pH 8.2 and 0.5 μ M at pH 6.8 for activated kinase as compared to 3 μ M at pH 8.2 and an indeterminate value at pH 6.8 for nonactivated kinase. Direct binding studies of calcium by phosphorylase kinase were used to determine the dissociation constants and minimal molar binding weight. They reported the dissociation constants were in the same range as the K_m values and 24 moles of calcium were tightly bound per mole of enzyme. It was also shown that inactive phosphorylase kinase is completely inhibited by fresh preparations of sarcoplasmic reticulum as well as EGTA.

A recent study of the calcium binding properties of nonactivated phosphorylase kinase at pH 6.8 was made using more sensitive calcium determination techniques (23). The enzyme was found to bind 12 moles of calcium per mole of enzyme with a K_D of 1.7×10^{-8} M, four moles with a K_D of 6×10^{-7} M and 36 moles with a K_D of 2.6×10^{-5} M when using a low ionic strength buffer. When the dissociation constants were determined in a buffer with an ionic strength similar to that found in muscle (in the presence of 180 mM NH_4Cl or 60 mM $(\text{NH}_4)_2\text{SO}_4$) only a single set of eight binding sites were found with a K_D of 1.8×10^{-8} M. When the studies were done in a buffer containing 155 mM NH_4Cl and 10 mM MgCl_2 , the calcium affinity of these sites was reduced to a K_D of 3.3×10^{-7} M indicating competition between Ca^{2+} and Mg^{2+} . Additionally, 10 mM Mg^{2+} induces a set of four new calcium binding sites which show positive cooperativity with a half saturation constant of 2.8×10^{-6} M. The authors point out that only two sets of calcium binding sites are apparent when determined under conditions of ionic

strength and Mg^{2+} concentration similar to that used in the enzymic activity assay (the eight sites with a K_D of 3.3×10^{-7} M and the four showing positive cooperativity) and one or both of them may be responsible for the stimulation of activity by calcium.

The phosphorylase kinase reaction

Phosphorylase kinase phosphorylates phosphorylase and itself in the autocatalytic reaction. The TN-I component of troponin is the only other protein known to be phosphorylated by phosphorylase kinase at a significant rate. The kinetic parameters of the phosphorylase b conversion reaction have been determined for nonactivated kinase and kinase activated by phosphorylation. The differences in the activities of the two forms relates primarily to a higher affinity for the substrate, phosphorylase b, by the activated enzyme form. Krebs, et al. (8) reported the K_m for phosphorylase was reduced 2.3 fold at pH 8.2 and 3.4 fold at pH 7.5 by activating the enzyme. The K_m for MgATP was not significantly changed by activation. A more recent study (24) using cyclic-AMP dependent protein kinase activated phosphorylase kinase reported the K_m for phosphorylase to be 12 μ M and the K_m for MgATP to be 120 μ M with a V_{max} of 15.6 μ moles/min/mg when determined at pH 7.6. The TN-I component of troponin is phosphorylated by kinase at 1/15th of the rate found for phosphorylase (25). The K_m for TN-I is very low, 4×10^{-6} M, and may be a physiological substrate. The pH dependence of the TN-I phosphorylation reaction was unusual. Both nonactivated and protein kinase activated phosphorylase kinase had an identical

response to changes in pH and the pH 6.8/8.2 activity ratio for both enzyme forms was 0.7. The pH 6.8/8.2 activity ratios normally found for nonactivated and activated phosphorylase kinase using phosphorylase b as substrate are approximately 0.04 and 0.38 respectively.

Reports of dissociation of phosphorylase kinase

Graves, et al. (19) reported that trypsin activated phosphorylase kinase can be dissociated by incubating the enzyme with 20 mM ATP at 5⁰. Essentially all of the 22S trypsinized kinase was dissociated to a 13S component after incubation with ATP for 18 hours and the dissociated species was catalytically active. The molecular weight of the 13S form was 350,000 as determined by sedimentation equilibrium. The dissociation process was dependent on many factors including time, pH, temperature and enzyme concentration and could not be reversed. Trypsinized phosphorylase kinase was shown to undergo further dissociation to produce catalytically active 9S and 6S species using analytical sucrose density gradient centrifugation techniques. Disc gel electrophoresis in the presence of SDS showed that the γ subunit was present in the catalytically active 6S fraction and the α and β subunits were absent. They were unable to establish that the γ subunit was the catalytic subunit of phosphorylase kinase because of contamination of the 6S fraction by other polypeptides which were trypsin degraded products of the α and/or β subunits. Graves, et al., were unable to dissociate nonactivated or protein kinase activated phosphorylase kinase using the conditions found effective in dissociating trypsin activated phosphorylase kinase.

Dissociation of phosphorylase kinase has been shown by frontal analysis gel filtration on Sepharose columns (26). Crude extracts were prepared from fresh minced rabbit muscle, applied to a calibrated column and the eluted fractions assayed for phosphorylase kinase and phosphorylase activities. Phosphorylase kinase and phosphorylase coeluted with an apparent molecular weight of 850,000 indicating dissociation of the 1.3×10^6 dalton native phosphorylase kinase and formation of a dissociated phosphorylase kinase-phosphorylase complex. Nearly the same result was obtained when a mixture of purified enzymes was used. Analysis of the activity elution profile indicated a dissociated phosphorylase kinase-phosphorylase complex coeluting with a molecular weight of 750,000. The authors were not able to explain why the molecular weight of the protein complex was dependent on the enzyme source. These results indicate phosphorylase kinase had dissociated to molecular weight forms of less than 650,000 depending upon the stoichiometry of the kinase-phosphorylase complex.

Phosphorylase kinase from the primitive vertebrate Squalus acanthias (Pacific dogfish) has been studied and many of its properties are similar to the rabbit enzyme including the subunit structure and the susceptibility of the α and β subunits to proteolytic degradation by trypsin (27). Two major differences which indicate dogfish kinase may be less highly regulated than the rabbit skeletal muscle enzyme are that the pH 6.8/8.2 activity ratio of the native enzyme is 0.5 and none of the subunits were found to be susceptible to phosphorylation. The amino acid composition of the γ subunit has been

determined and found to be identical to dogfish actin within the limits of error of the procedure used with only one distinct difference; whereas dogfish actin contains one 3-methyl histidine, the γ subunit had none. It should be mentioned that similarities between the amino acid composition of actin and the γ subunit of rabbit muscle phosphorylase kinase are not apparent. The authors presented limited evidence that the α subunit of dogfish phosphorylase kinase has a regulatory function and the β subunit was the catalytic subunit. Rapid activation of the enzyme during trypsinization was concomitant with the disappearance of the α subunit as determined by disc gel analysis in the presence of SDS indicating to these authors a regulatory role for the α subunit. Further proteolysis of the enzyme resulted in the degradation of the β subunit and loss of enzyme activity while the γ subunit was resistant to trypsin attack. It should be noted that during the trypsinization of rabbit muscle phosphorylase kinase a similar activation and sequential degradation of the α subunit and β subunit is seen. Partially degraded preparations of dogfish phosphorylase kinase were chromatographed on a Biogel column and an active fraction containing only the β subunit was recovered as determined by disc gel electrophoresis in the presence of SDS. The single gel was the only evidence presented to support their conclusion that the β subunit is the catalytic subunit of dogfish phosphorylase kinase and a more complete paper describing this enzyme is eagerly awaited.

New approaches to understanding phosphorylase kinase

Highly purified phosphorylase kinase has been available for study for several years but progress on understanding its mechanisms of action and regulation at a molecular level has been slow because of the complexity of the system. Until recently, the only substrate readily available for use in studying phosphorylase kinase has been phosphorylase b. This has imposed severe restrictions on the type of experimentation that could be done as well as complicated interpretations of experimental results. Synthetic peptides which will serve as substrates for phosphorylase kinase have become readily available for studying phosphorylase kinase-substrate interactions. A tetradecapeptide representing the N-terminal region of phosphorylase has been shown to be a good model substrate and allowed experimentation to be done that is impossible using the more complex substrate, phosphorylase (24, 28). Determination of the reaction mechanism was done using the tetradecapeptide as substrate and another peptide as its competitive inhibitor. The results using the peptides, ATP and an ATP analogue indicated a random bi-bi reaction mechanism for phosphorylase kinase (24). The tetradecapeptide and similar peptides with changes in the length and amino acid sequence were used in determining the substrate specificity of the kinase reaction (28). The results indicated that important determinants for phosphorylase kinase substrate recognition reside in the linear sequence of the tetradecapeptide. The amino acids near the convertible seryl residue (Lys-Gln-Ile-Ser-Val-Arg) were found to be particularly important for kinase recognition of substrates.

Studies of effectors of the phosphorylase kinase reaction have been difficult to interpret using phosphorylase as the substrate. A particular effector may influence the reaction by binding to phosphorylase or kinase or both. The use of a less complex substrate, the tetradecapeptide, has simplified studying the effect of modifiers of the phosphorylase kinase reaction. For example, glycogen was found to stimulate the phosphorylase kinase reaction by decreasing the K_m for phosphorylase 10 fold (8). When the effect of glycogen on the kinase reaction was examined using the tetradecapeptide as substrate, a small increase in the V_{max} and no change in the K_m for peptide was observed, indicating glycogen is mainly a substrate directed effector when phosphorylase is used as substrate (24). The use of peptide substrates in studying phosphorylase kinase has allowed studies to be done on enzyme-substrate complexes that were previously not feasible.

Another complexity inherent to the study of native phosphorylase kinase is that the enzyme is a dodecamer composed of three different subunits. The function of none of the subunits is known with certainty. The enzyme is highly regulated and its in vivo and in vitro enzymatic activity is changed in response to covalent modification and effectors. The state of phosphorylation of the enzyme has a profound effect upon its activity. Preparations of nonactivated phosphorylase kinase are found to contain variable amounts of endogeneous phosphate (29), which may affect the properties of the enzyme. Phosphorylase kinase is totally inactive in the absence of calcium, is stimulated by preincubation with its substrate (13), and is autoactivated during the catalytic

reaction (14). Progress on understanding the regulatory features of kinase has been impeded by the complexity of the enzyme itself.

Understanding the mechanisms of action and regulation of kinase would be simplified by having purified subunits of phosphorylase kinase available for study. The available evidence suggests that at least 2 subunits of the enzyme have important regulatory features and at least one subunit must possess catalytic activity.

The purpose of this work is to identify, isolate and purify the catalytic subunit of the enzyme. Characterization of the purified catalytic subunit should provide insights into mechanisms of regulation of the native enzyme. The substrate specificity of the purified catalytic subunit should be examined and compared to that of the native enzyme. This may indicate whether a regulatory or catalytic subunit is responsible for the high degree of substrate specificity found for the native enzyme. Long range goals implicit to the project are the purification of all subunits of phosphorylase kinase. Studies using the individual purified subunits and recombinant forms should provide new and important information about the mechanisms of action and regulation of phosphorylase kinase.

MATERIALS AND METHODS

Phosphorylase b kinase was prepared from rabbit muscle by the method of DeLange, et al. (30) as modified by Hayakawa, et al. (10). Phosphorylase b was prepared as described by Fisher and Krebs (31) except that 30 mM 2-mercaptoethanol was substituted for cysteine. Residual AMP was removed by treatment with acid washed Norite A. Phosphorylase was assayed in the direction of glycogen synthesis according to the method of Illingworth and Cori (32). Protein concentrations of the purified enzymes were determined spectrophotometrically using either $A_{280\text{ nm}}^{1\%}$ of 12 (10) or 13 (33) for phosphorylase kinase and phosphorylase respectively. Other protein determinations utilized the Comassie Brilliant Blue G-250 microprotein assay described by Bradford (34). Often the samples contained ATP which increased the background slightly but the standard curves were linear. Phosphorylase kinase was used as the standard for the assay.

Phosphorylase kinase was assayed as described (6) except 0.1 mM CaCl_2 was included in the reaction mixture unless noted otherwise. The activity was usually determined enzymatically by measuring phosphorylase a production. Phosphorylase kinase activity was determined in some experiments using the same assay conditions as described above except that the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into phosphorylase was followed using the filter paper assay (35). Reactions were terminated by pipetting 25 μl of reaction mixture on Whatman ET 31 chroma filter paper (1.5 x 2 cm rectangles) and washing the papers as described. The papers were counted in 10 ml of

toluene based scintillation fluid in Packard scintillation counters at 1.3% gain. After counting, the papers were extracted from the vials and the same vial could be used several times. For the sucrose density gradient centrifugation experiments, activity determinations were done using nearly the same assay conditions as described above. The ATP from the gradient served as the source of nonradioactive ATP and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added to the reaction mixtures. Aliquots of the gradient fractions were diluted 6 fold in 40 mM β -glycerophosphate, 30 mM 2-mercaptoethanol, pH 6.8 and provided the source of unlabeled ATP (2.8 mM final concentration in the reaction mixture) and enzyme to initiate the kinase reaction.

Sucrose density gradient centrifugations were done using a Beckman 50.1 swinging bucket rotor in a Beckman L-5 ultracentrifuge. The gradients were fractionated automatically from the top by connecting a Buchler auto densiflow apparatus which sampled the gradients at the miniscus to a fraction collector. Glycogen phosphorylase b and hemoglobin were used as markers to estimate the S values of the fractions.

Blue Dextran-Sepharose was prepared essentially by the method of Ryan and Vestling (36). The procedure was modified slightly by including sodium phosphate in the cyanogen bromide reaction mixture to aid in controlling the pH. Fifty grams of packed Sepharose 4B was suspended in 50 ml of 0.25 M Na_2PO_4 , pH 11. Fifteen grams of cyanogen bromide was added and the pH was maintained near pH 11 with 10N NaOH and the temperature near 20° by the addition of small amounts of crushed ice. After 10 minutes reaction time, the reaction was quenched by the addition of a large volume of crushed ice. The

procedures described by Ryan and Vestling were followed for washing the resin and coupling the Blue Dextran 2000 to the activated Sepharose.

Acrylamide gel electrophoresis in the presence of SDS was done using the alkaline buffer system described by Hayakawa, et al., (11). Gels contained 7.5% acrylamide monomer and 0.13% bis-acrylamide. The buffer used for making the gels contained 100 gm Tris, 10 gm EDTA, 3.8 gm boric acid and 1 gm SDS per 500 ml, pH 9.3. The gel buffer was diluted 1:1 with water and used as the running buffer. Electrophoresis was performed at 6.5 ma per gel for 2.5 hours. Gels were stained overnight in a 0.125% Comassie Brilliant Blue, 45% methanol, 9.2% acetic acid solution. The gels were destained for 20 minutes electrophoretically in 7.5% acetic acid and allowed to complete destaining by diffusion in a 7.5% acetic acid, 10% methanol solution.

Often the protein samples to be analyzed by acrylamide gel electrophoresis were dilute and contained high concentrations of salts. This necessitated using special procedures in their preparation for electrophoresis and the procedures were followed for all samples. The samples were extensively dialyzed against a buffer containing 8 M urea, 10 mM sodium phosphate, pH 7.0, 30 mM 2-mercaptoethanol, 2 mM EDTA to denature the protein and remove salts from the samples. They were concentrated to approximately 0.1 ml against Aquacide powder and dialyzed again in the urea buffer. SDS and 2-mercaptoethanol was added at a final concentration of 1% and the samples were heat treated at 65° for 30 minutes. 2.5 µl of bromophenyl blue tracking dye (0.03%) and

2.5 μ l of 2-mercaptoethanol were added and the samples were applied to the gel and electrophoresed.

Peak integration of the acrylamide gels was performed using a System I Computing Integrator (Spectra-Physics) that received input directly from a Zeiss PMQ II Spectrophotometer having a modified scanning attachment to hold the cuvettes horizontally. Densitometer tracings were made using a Honeywell Electronik 194 recorder added in series with the spectrophotometer and computing integrator. The gels were scanned at 550 nm. The computing integrator was programmed to correct for baseline drift by establishing baseline before and after each peak. In the case of fused peaks, baseline was established before and after the fused peak group and horizontal or trapazoidal corrections were applied to each peak.

Rabbit muscle lactate dehydrogenase was obtained from Cal Biochem, pig heart malic dehydrogenase from Sigma Chemical Co. Pig heart aspartate transaminase was a gift from D.E. Metzler's research group, hemoglobin from M.A. Rougvie, cyclic-AMP dependent protein kinase activated phosphorylase kinase from L.A. Tabatabai, all of Iowa State University. LDH and MDH were assayed as described in the Worthington Enzyme Manual (37) and AAT by the method of Furbish, et al. (38).

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was prepared by the method of Glynn and Chappell (39). The ATP used in the sucrose density gradients and for elution of Blue Dextran-Sepharose columns was the Grade II disodium salt of ATP obtained from Sigma Chemical Co. Grade I was used elsewhere. All other materials used were commercially available.

RESULTS

Factors that promote dissociation of nonactivated phosphorylase kinase

ATP has been shown to dissociate trypsin activated phosphorylase kinase to catalytically active 13S, 9S and 6S fragments but did not affect the subunit structure of native phosphorylase kinase (19). The use of ATP and chaotropic salts to dissociate phosphorylase kinase while retaining catalytic activity was examined. Nonactivated phosphorylase kinase was incubated with possible dissociating agents at 0° for several hours under a variety of conditions and its activity at pH 6.8, pH 8.2 and pH 8.2 in the presence of EGTA was monitored. When unusual changes in the activities were seen, sucrose density gradient centrifugations of incubated kinase samples were done. The gradients were fractionated and analyzed for catalytic activity of dissociated phosphorylase kinase species. The experimental conditions used in the centrifugations such as time and speed of centrifugation would result in proteins with S values from 8S to 14S migrating near the middle of the gradient. Proteins with large S values such as the 23S native phosphorylase kinase would be found at the bottom of the tube after centrifugation. Preliminary studies using ATP as a potential dissociating agent indicated the concentration of the nucleotide in the incubation mixture had to be greater than 25 mM to see any change in the activity of the enzyme that differed from control values and 100 mM ATP, the highest concentration used, was more effective than 50 mM ATP. Density gradient centrifugation of inactive kinase that had been incubated with 100 mM ATP for 33 hours at 0° produced two

kinase activity peaks located near the middle of the gradient, fractions 5 through 10 (Figure 1). Essentially no kinase activity was found in the fractions near the top of the gradient. Only a small amount of activity was found in the bottom fraction of the gradient indicating either near complete dissociation of kinase or the native enzyme had pelleted on the bottom of the tube. The S value of the peak found at fractions 5 and 6 was approximately 8S and the peak at fraction 10 was 14S indicating incubation of phosphorylase kinase with ATP will dissociate the native enzyme. The effect of pH and EGTA on the activity of the dissociated phosphorylase kinase fragments found in fractions 5 and 10 was tested. The pH 6.8/8.2 activity ratio was 0.44 and 0.32 for fractions 5 and 10 respectively. The inclusion of 0.83 mM EGTA in the assay reaction mixture caused no inhibition of activity and the reaction progress curves were linear.

Other experiments (not shown) using ATP as the dissociating agent and sucrose density gradient centrifugation analysis were done to determine the best conditions to use for dissociating phosphorylase kinase. The use of MES buffer resulted in more dissociated phosphorylase kinase activity being found in the gradients than when HEPES, imidazole or Tris/ β -glycerophosphate buffers were used. Little or no activity was found throughout the sucrose gradient if ATP was not present in the gradient. No effect of pH was seen when the pH of the incubation mixture and/or gradient was pH 6.8 or pH 8.6. Cyclic-AMP dependent protein kinase activated phosphorylase kinase was found to be less susceptible to ATP dissociation than nonactivated kinase. Centrifugation

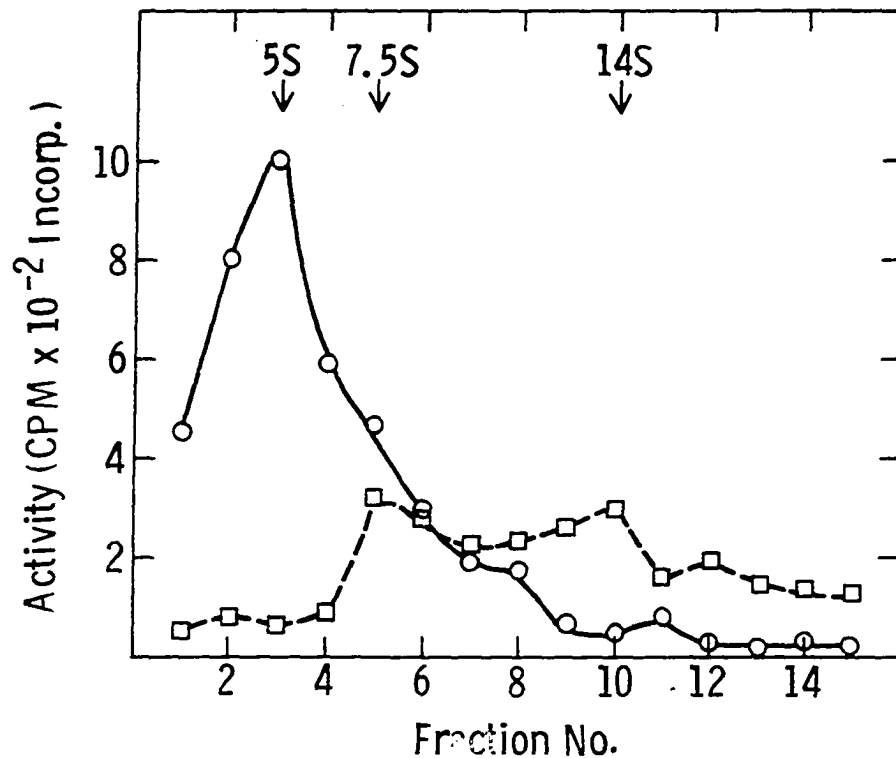


Figure 1. Sucrose density gradient centrifugation of inactive phosphorylase kinase that had been incubated with either ATP or LiBr. Kinase (0.45 mg/ml) was incubated in a solution containing either 2.5% sucrose, 0.5 mM EDTA, 0.5 mM CaCl_2 , 34 mM β -glycerophosphate, 32 mM Tris, 100 mM ATP, pH 8.6² at 0° for 33 hours or 2.5% sucrose, 2.5 mM β -glycerophosphate, 0.5 mM EDTA, 50 mM MES, 1 M LiBr, pH 7.0 at 0° for 9 hours. 0.09 mg. of kinase was applied to a 5 ml sucrose gradient (5 to 20%) containing 31 mM β -glycerophosphate, 31 mM Tris, 100 mM ATP, 10 μM CaCl_2 , pH 8.6 and centrifuged at 4° for 11 hours at 40,000 RPM.² The gradients were fractionated and assayed as described in materials and methods. Phosphorylase kinase incubated with ATP, (□); with LiBr, (○).

of activated phosphorylase kinase that had incubated with ATP resulted in very little kinase activity being found in fractions having S values of 14S or less. Therefore, nonactivated phosphorylase kinase was used in all other experiments. No conditions were found using ATP as the dissociating agent that produced active fragments with an S value of less than approximately 8S indicating that full dissociation of the holoenzyme to individual subunit forms probably had not occurred.

Chaotropic salts as dissociating agents

Two properties found for ATP dissociated phosphorylase kinase have also been observed for nonactivated kinase after incubation with NaNO_3 . An earlier report (40) has shown that after incubating nonactivated phosphorylase kinase with near molar concentrations of NaNO_3 , the pH 6.8 activity was stimulated 5 fold and the lag normally found for nonactivated kinase was abolished. Attempts were made in the study to determine if 1M NH_4NO_3 or LiBr induced dissociation of kinase but the salts caused aggregation of the enzyme. The experiments using NaNO_3 , LiBr, and NH_4NO_3 were done at 30°.

The effect of incubating nonactivated phosphorylase kinase with near molar concentrations of the chaotropic salts at 0° was examined. The enzyme activity at pH 6.8, pH 8.2 and pH 8.2 in the presence of EGTA was determined for nonactivated phosphorylase kinase during incubation with 0.83 M LiBr, NaNO_3 or RbBr (Table 1). Incubation of kinase with LiBr and NaNO_3 resulted in the pH 6.8/8.2 activity ratio to increase to approximately 0.5 with almost complete loss of sensitivity to EGTA. Incubating kinase with LiBr appeared slightly more effective than

Table 1. Effect of 0.83 M chaotropic salt on the properties of phosphorylase kinase.^a

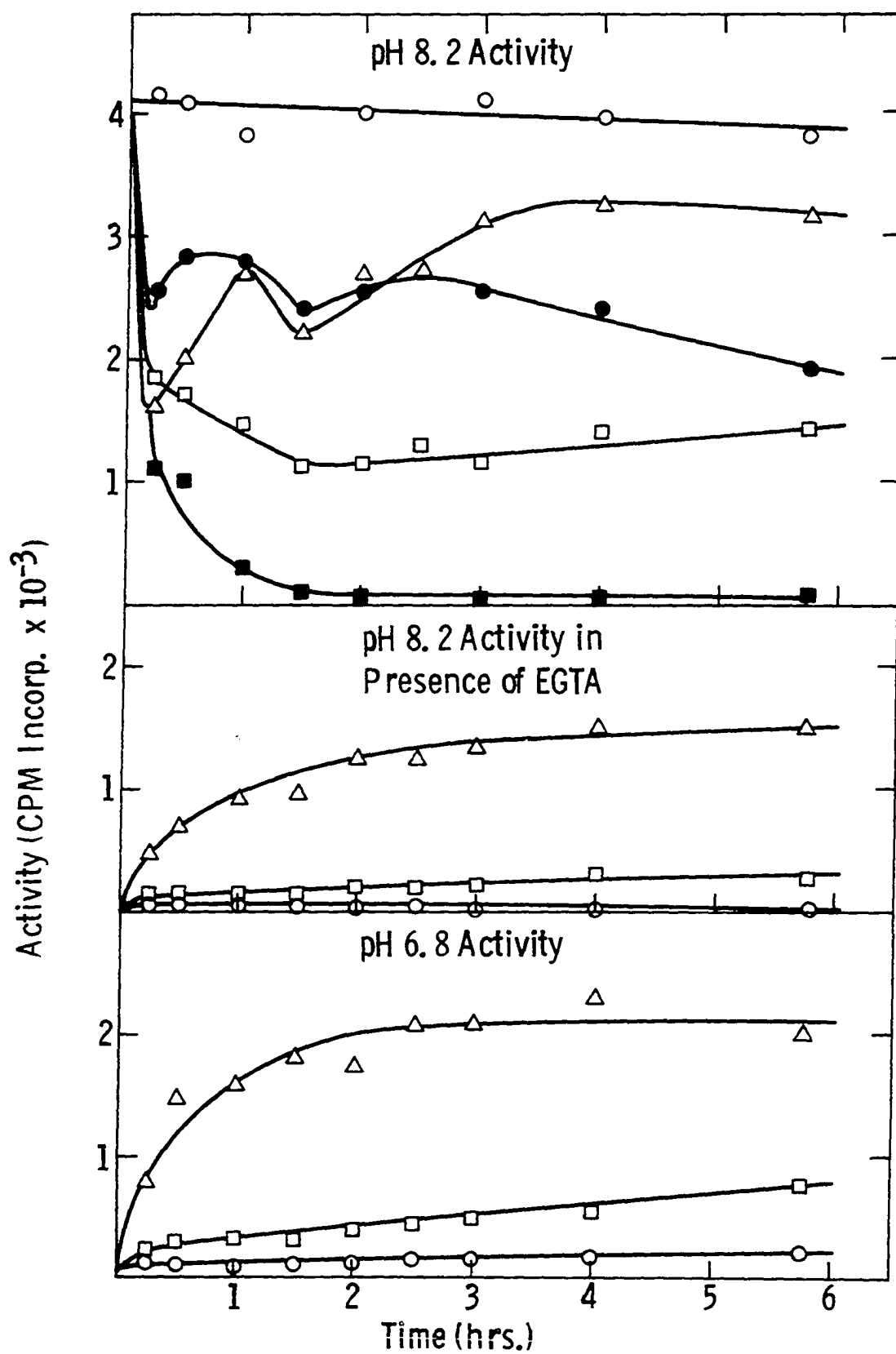
Time of Incubation	1/2 hour		4 hours		23 hours	
Salt	pH 6.8/8.2 Activity Ratio	Inhibition by EGTA	pH 6.8/8.2 Activity Ratio	Inhibition by EGTA	pH 6.8/8.2 Activity Ratio	Inhibition by EGTA
LiBr	0.33	61%	0.51	1%	0.54	3%
NaNO ₃	0.18	80%	0.39	32%	0.56	6%
RbBr	0.14	95%	0.10	87%	0.18	75%
H ₂ O	0.16	94%	0.15	92%	0.18	93%

^aInactive phosphorylase kinase was incubated at 0° with LiBr, NaNO₃ or RbBr. The incubation mixture contained kinase at 0.3 mg/ml, 0.83 M chaotropic salt, 42 mM MES, 1.2 mM EDTA, 1.7% sucrose and 1.7 mM β-glycerophosphate, pH 7.0. After 1/2 hour, 4 hours and 23 hours, aliquots of the incubation mixtures were diluted in 40 mM β-glycerophosphate, 30 mM 2-mercaptoethanol, pH 6.8 and assayed as described in methods and materials for activity at pH 6.8, pH 8.2 in the presence of 8.3 μM CaCl₂ and pH 8.2 in the presence of 83 μM EGTA.

with NaNO_3 in promoting the changes found to be characteristic of ATP dissociated phosphorylase kinase. Incubation with RbBr caused little change over control values. The effect of incubating inactive phosphorylase kinase with LiBr on the subunit structure is shown in Figure 1. Centrifugation of inactive kinase that had been incubated with 1M LiBr for 9 hours produced one catalytically active peak with an S value of approximately $5S$. The pH 6.8/8.2 activity ratio of fraction 3 of this experiment was 0.47 and 0.83 mM EGTA caused 50% inhibition of the pH 8.2 activity. Additional sucrose density gradient centrifugations of LiBr treated kinase were done to determine what effect time of incubation had on dissociation and to prepare samples for SDS disc gel analysis. If the incubation period with 1M LiBr was shortened to 2 hours, two activity peaks were seen (not shown). One peak had an S value of approximately $5S$ and a second peak was broad and ranged from $8S$ to $13S$. The results of a control sample that had been incubated using the same conditions except the incubation period was 7.5 hours were essentially the same as those shown in Figure 1. Only one kinase activity peak with an S value of approximately $5S$ was found in this gradient. Fractions containing the low molecular weight active phosphorylase kinase were analyzed using disc gel electrophoresis in the presence of SDS. All three subunits of phosphorylase kinase were found in these fractions indicating that although LiBr is an effective dissociating agent, preparative sucrose density gradient centrifugation would not be a suitable method for purification of the catalytic subunit of phosphorylase kinase.

The effect of incubating inactive phosphorylase kinase with various concentrations of LiBr on enzyme activity at pH 6.8, pH 8.2 and pH 8.2 in the presence of 0.1 mM EGTA was examined to determine the best concentration of LiBr to use in further dissociation studies (Figure 2). Incubation with 1.8 M LiBr resulted in a large loss of activity during the initial 1/2 hour which was mostly regained after 5.75 hours of incubation. The pH 6.8 activity steadily increased from 0.03 to 0.67 and the inhibition by EGTA steadily decreased from 99% to 52% when phosphorylase kinase was incubated with 1.8 M LiBr. Incubation with 3.6 M LiBr caused a rapid loss of activity and 2.7 M LiBr caused a significant loss that was not reversed during the incubation period. 0.9 M LiBr also caused a loss of pH 8.2 activity that was not regained and only small changes in the pH 6.8 and pH 8.2 in the presence of EGTA activities were seen. Also included in the figure is a control incubation mixture having no LiBr added. The results show that nonactivated phosphorylase kinase is stable during the incubation period with no loss of EGTA sensitivity or increase in the pH 6.8 activity. The criteria used to determine the best concentration of LiBr to use in further dissociation experiments were the increase in the pH 6.8/8.2 activity ratio, loss of sensitivity to EGTA and retention of total pH 8.2 activity. Over the LiBr concentration range examined, 1.8 M LiBr best satisfied these requirements. Experimentation to support a satisfactory explanation for the fluctuations in the pH 8.2 activities has not been done but they were consistently seen when phosphorylase kinase was incubated under conditions that caused dissociation including incubation

Figure 2. Effect of incubation with LiBr on the activities of phosphorylase kinase. Phosphorylase kinase (0.85 mg/ml) was incubated at 0° with 3.6% sucrose, 18 mM β -glycerophosphate, 0.73 mM EDTA, 0.73 mM CaCl_2 , 91 mM MES, pH 7.0 and varying concentrations of LiBr. No LiBr added (\circ); 0.9 M LiBr (\square); 1.8 M LiBr (\triangle); 2.7 M LiBr (\bullet); 3.6 M LiBr (\blacksquare). At various times aliquots of the incubation mixture were diluted in 40 mM β -glycerophosphate, 30 mM 2-mercaptoethanol, pH 6.8 and assayed for activity at pH 6.8, pH 8.2 and pH 8.2 in the presence of 0.1 mM EGTA.



with 100 mM ATP. The specific activity of phosphorylase kinase after an overnight incubation with 1.8M LiBr was usually about 95,000 units per mg. Nearly the same specific activities were found after incubation for an aged phosphorylase kinase preparation that had a specific activity of less than 50,000 units per mg and fresh preparations with specific activities of approximately 100,000 units per mg.

Purification of the catalytic subunit

Blue Dextran-Sepharose has been used as an affinity chromatographic gel for several kinases and dehydrogenases (41) and was found to bind inactive phosphorylase kinase as well as LiBr treated kinase. The results shown in Figure 3 indicate that the gel can be used for partial purification of the catalytic subunit of phosphorylase kinase. In this experiment the specific activity of the LiBr treated kinase applied to the Blue Dextran-Sepharose was 89,000 units per mg. The specific activity of the peak fractions eluted by a linear ATP gradient was increased approximately 4 fold. Fifty-four percent of the total activity applied to the column was recovered in the three fractions having a specific activity greater than 350,000 units per mg and the total activity recovered throughout the gradient was 88% of that applied. Only 40% of the applied protein was recovered in the eluted fractions. Disc gel analysis in the presence of SDS of fractions from similar experiments (not shown) showed an enrichment of the γ subunit for the peak fractions suggesting it may be the catalytic subunit of phosphorylase kinase. The peak fractions were contaminated by the α subunit which tended to elute after the activity peak and a

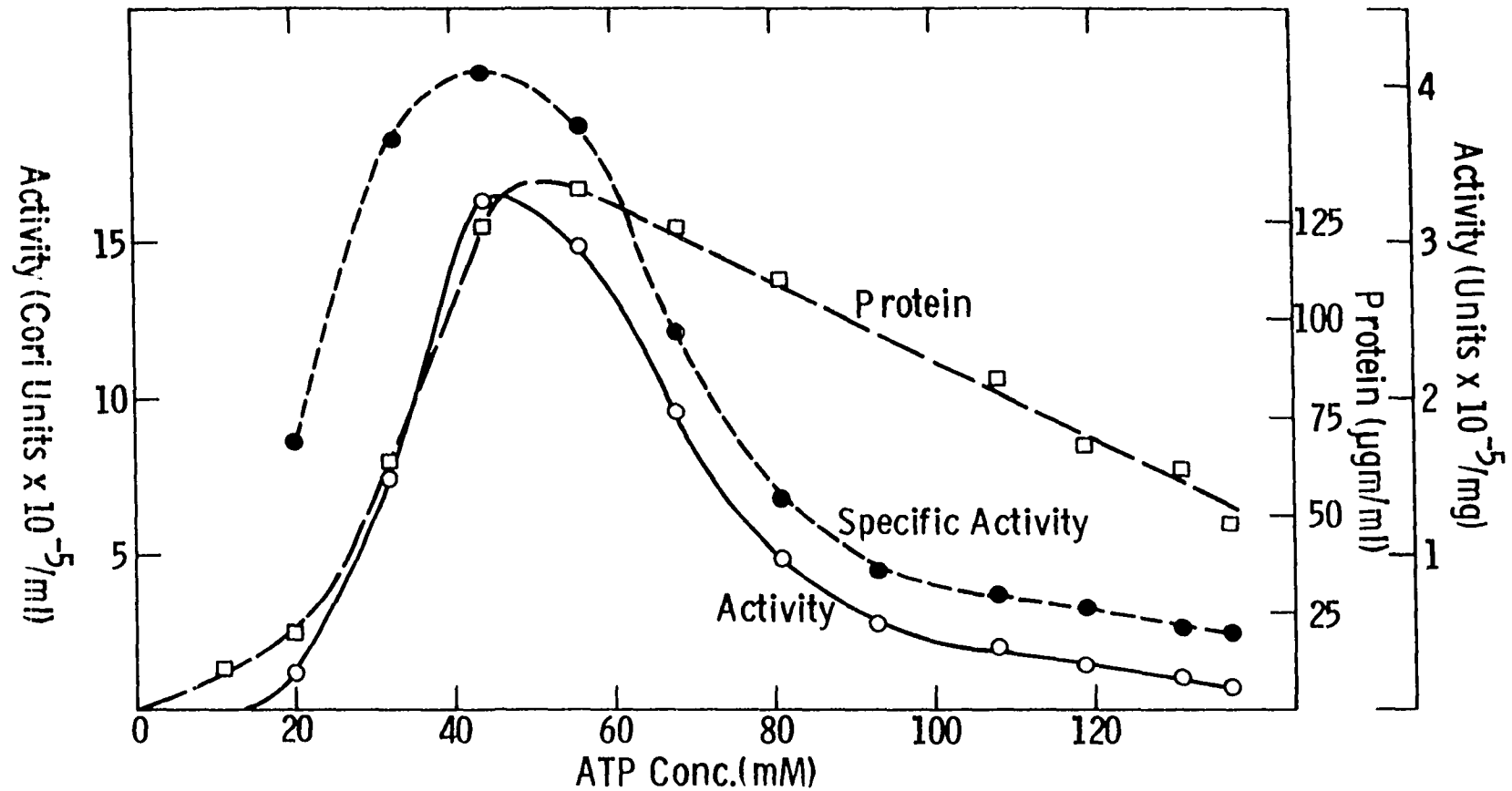


Figure 3. Affinity chromatography of LiBr treated phosphorylase kinase. Inactive phosphorylase kinase was incubated with LiBr for 13 hours at 0° in a mixture containing kinase at 1.1 mg/ml, 4.6% sucrose, 0.91 mM EDTA, 0.91 mM CaCl_2 , 23 mM β -glycerophosphate, 91 mM MES, and 1.8 M LiBr, pH 7.0. The enzyme was chromatographed on a 3 ml Blue Dextran-Sephadex column equilibrated with 100 mM MES, 0.5 mM CaCl_2 , 30 mM 2-mercaptoethanol, pH 7.0. 2.2 ml of incubation mixture was diluted 10 fold in the equilibration buffer and applied to the column. The column was then washed with 3 column volumes of buffer and eluted with a 16 ml linear ATP gradient in the equilibration buffer. 0.65 ml fractions were collected. The enzyme activity (\circ), protein concentration (\square) and specific activity (\bullet) are plotted against the concentration of ATP in the eluted fractions.

protein assumed to be phosphorylase based on SDS disc gel mobility which tended to elute ahead of the activity peak. This protein has been shown to be a minor contaminant of phosphorylase kinase preparations (9). Other ligands and salts were tested as eluting agents of LiBr treated kinase bound to Blue Dextran-Sepharose. 1 M NaCl and the kinase substrate MgATP (a linear MgATP gradient from 10 mM MgAc₂/0 mM ATP to 110 mM MgAc₂/100 mM ATP) were ineffective in eluting the kinase activity from the column. Elution with an ADP gradient resulted in the same activity and protein elution profile as found when ATP was used. Ninety-nine percent of the activity applied to the affinity column could be eluted by a linear LiBr gradient. The activity eluted in one symmetrical peak at 0.65 M LiBr but the protein elution profile could not be determined because of interference in the protein assay by the high concentrations of the salt. Disc gel analysis in the presence of SDS of the fractions eluted by LiBr showed more contamination of the γ subunit by other proteins than was found when ATP was used as the eluting agent.

Near pure preparations of the γ subunit required a three step isolation scheme. The native phosphorylase kinase was dissociated by incubation with 1.8 M LiBr for 12 to 18 hours at 0⁰, partially purified by G-150 Sephadex molecular sieve chromatography and both concentrated and further purified by Blue Dextran-Sepharose affinity chromatography.

Figure 4 shows the activity and elution profiles of LiBr treated phosphorylase kinase chromatographed on G-150 Sephadex. Substantial

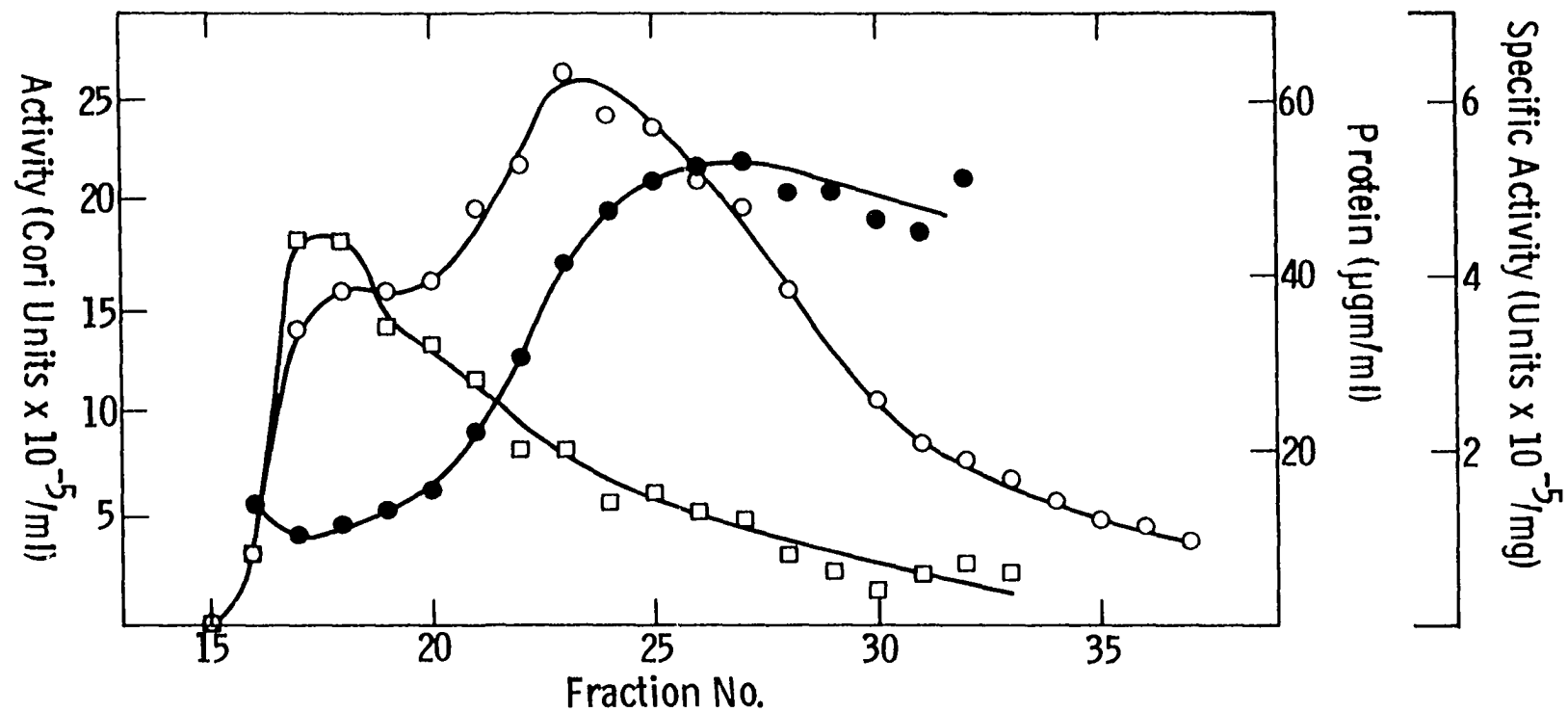
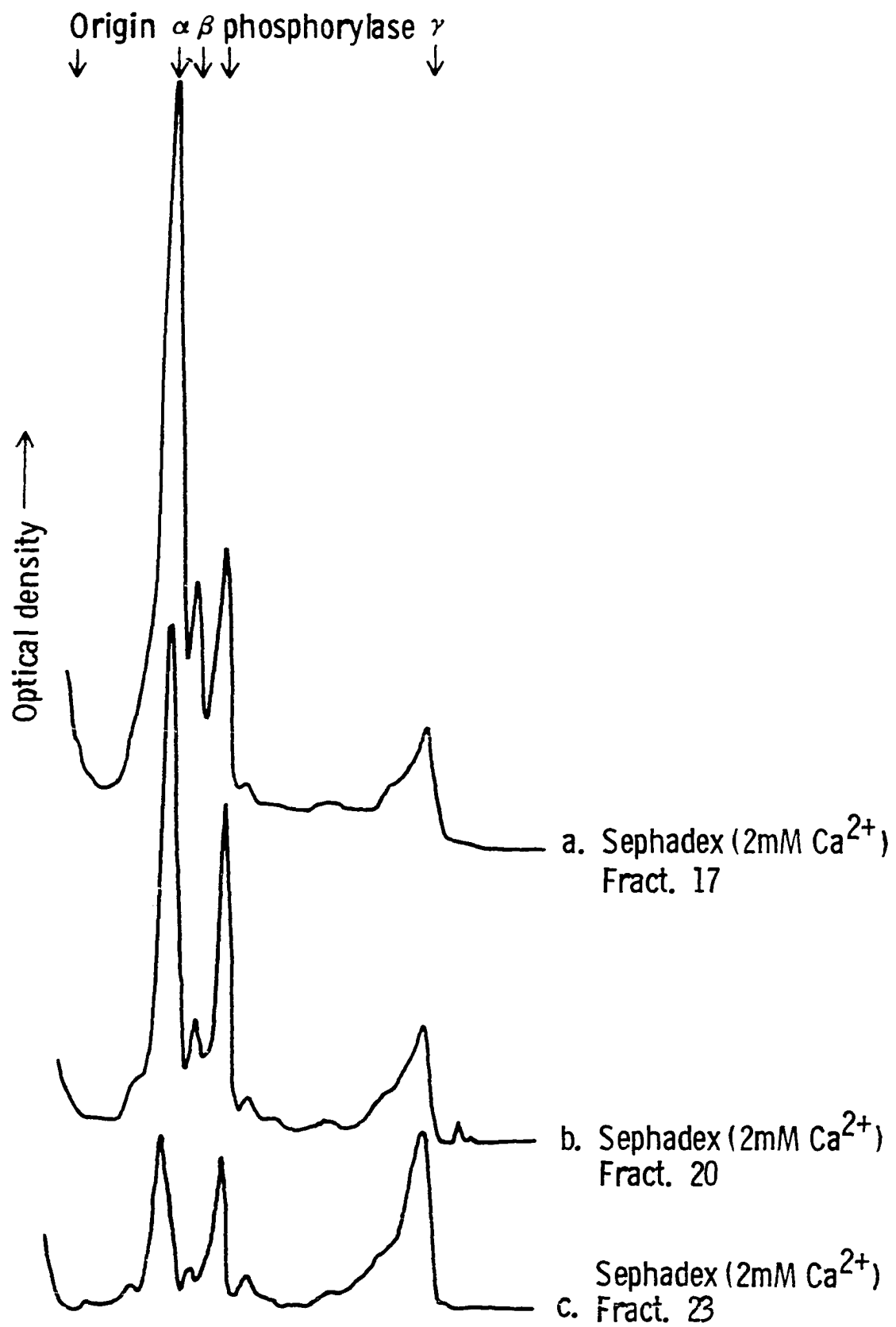


Figure 4. Sephadex chromatography of LiBr treated kinase. Inactive phosphorylase kinase was incubated with LiBr for 18 hours at 0° in an incubation mixture as described in the legend for Figure 3, except kinase was 0.91 mg/ml. 2.7 ml of the incubation mixture was applied to a G-150 Sephadex column (1.7 \times .32 cm) equilibrated with 100 mM MES, 0.5 mM CaCl_2 , 30 mM 2-mercaptoethanol, pH 7.0. 1.7 ml fractions were collected. Kinase activity at pH 8.2 (\bigcirc), protein concentration (\square), and calculated specific activity (\bullet) are plotted.

purification of the catalytic subunit eluting in the retarded activity peak would be predicted from the increased specific activity of these fractions. Sixty-six percent of the applied activity was recovered in the eluted fractions and 2/3 of the recovered activity was found in fractions 23 through 32. A second Sephadex chromatography experiment was done using the same conditions except that the CaCl_2 concentration in the column buffer was increased to 2 mM. The activity and protein elution profiles of the two experiments were identical and disc gel analysis in the presence of SDS of selected fractions from the second experiment and fractions 30 plus 31 from the first was done. Densitometric tracings of the polycrylamide gels and a native phosphorylase kinase gel are shown in Figure 5. The molar ratios of the α subunit, β subunit, and phosphorylase to the γ subunit were calculated from integration of gel densities and are presented in Table 2. The stoichiometry of the subunits for native phosphorylase kinase was found to be $\alpha_{1.05}$, $\beta_{1.16}$, $\gamma_{1.00}$ which agreed closely with the results published by Cohen (9). The molecular weights of $\alpha = 145,000$, $\beta = 128,000$, $\gamma = 45,000$, and phosphorylase = 100,000 were used in the molar ratio calculations. Substantial purification of the γ subunit for fractions eluting after the major protein peak is indicated by the densitometric tracings of gels for these fractions. Associated with the purification of the γ subunit is a concomitant increase in specific activity. The α subunit eluted mainly in or near the void volume, fractions 17 through 20. Very little β subunit was found in any of the fractions and presumably it either precipitated in or bound

Figure 5. Densitometric tracings of SDS acrylamide gels of phosphorylase kinase subunits partially purified on a Sephadex column. The LiBr incubation conditions are described in the legend to Figure 3 and the G-150 Sephadex chromatography procedures are described in the legend to Figure 4 except the CaCl_2 concentration in the buffer was increased to 2 mM as discussed in the text.

Tracings 5a - 5e are of gels of fractions eluted from a Sephadex column with an elution profile nearly identical to that shown in Figure 4. Tracing 5f is of a gel of fraction 29 plus 30 eluted from the Sephadex column depicted in Figure 4. The entire fraction or combined fractions were denatured, concentrated and electrophoresed as described in Materials and Methods. Tracing 5g is of a gel of native phosphorylase kinase which was prepared for electrophoresis using the same procedures.



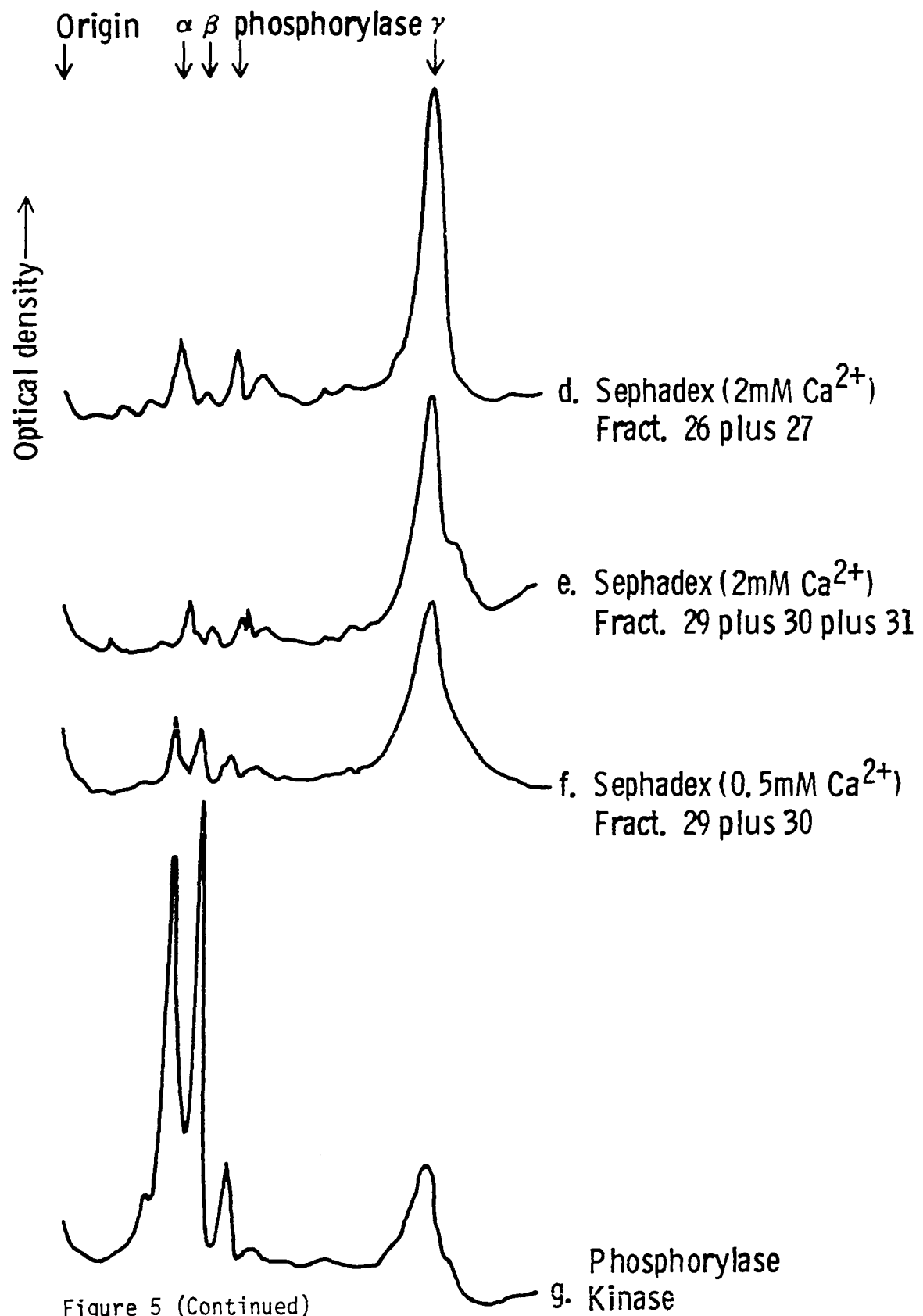


Table 2. Molar ratios of α subunit, β subunit and phosphorylase to γ subunit.^a

Column	Fraction No.	α	β	phos.	γ
Sephadex (2 mM CaCl_2)	17	2.91	0.51	0.85	1
	20	0.56	0.03	0.38	1
	23	0.13	<0.03	0.12	1
	26 + 27	0.03	<0.03	0.03	1
	29 + 30 + 31	0.04	<0.03	0.04	1
Sephadex (0.5 mM CaCl_2)	29 + 30	0.04	0.03	0.03	1
Blue Dextran- Sephacrose	11 + 25 mM ATP	<0.03	<0.03	0.14	1
	33 + 41 mM ATP	0.03	0.04	0.05	1
	49 + 57 mM ATP	0.05	0.03	0.03	1
	63 + 72 mM ATP	0.10	0.04	0.05	1
Untreated native phosphorylase kinase		1.05	1.16	0.08	1

^aThe molar ratios were calculated by integration of the gel densities of SDS acrylamide gels depicted in Figures 5 and 7 using the procedures described in the text.

tightly to the gel matrix. The phosphorylase kinase activity peak was fraction 23. The densitometric tracing of the gel for this fraction and the subunit molar ratio calculations show that the peak fraction is nearly devoid of the β subunit. The γ subunit has become the major protein present but the α subunit and phosphorylase are present in significant amounts. More pure fractions of the γ subunit were found in the later fractions eluted from the column.

The molecular weight of the retarded peak was estimated by chromatographing a LiBr treated kinase sample on a G-150 Sephadex column calibrated with LDH, AAT and MDH. In an experiment nearly identical to that described in the legend for Figure 4, 0.22 mg of aspartate transaminase was added to the LiBr treated kinase incubation mixture just prior to sample application to the calibrated column and the activity elution profiles for both kinase and aspartate transaminase were determined. The retarded phosphorylase kinase activity peak eluted 1/2 fraction behind the transaminase activity peak indicating the smallest molecular weight form with phosphorylase kinase activity eluting from the column was approximately 86,000.

Although molecular sieve chromatography of LiBr treated kinase results in substantial purification of the γ subunit, the protein solutions are extremely dilute and an additional step is necessary for concentrating the enzyme. Blue Dextran-Sepharose affinity chromatography was chosen because it can both further purify and concentrate the catalytic subunit. In the experiment shown in Figure 6, fractions with a specific activity greater than 300,000 from the Sephadex column

depicted in Figure 4 were applied to a Blue Dextran-Sepharose column and eluted with an ATP gradient. The effectiveness of using these two columns in series to purify the catalytic subunit can be seen in Figures 6 and 7 and in Table 2. The specific activity has increased from 89,000 units per mg for the phosphorylase kinase incubated with LiBr to over 500,000 units per mg for the peak fractions. The purification of the γ subunit from the other subunits is clearly shown in the densitometric tracing of the peak fractions (7b and 7c) when compared to native phosphorylase kinase (7a). Quantitation of the gel densities shows that there is over 30 times more γ subunit than any other protein on the gel for the peak fractions. These data strongly suggest that the catalytic subunit of phosphorylase kinase is the γ subunit.

An experiment was done to determine the yield and purity of the γ subunit that can be obtained using the purification scheme on a larger scale. The procedures followed were nearly identical to those described in the legends to Figure 4 and 6 except the size of the incubation mixture and Sephadex column was increased 8 fold and the Blue Dextran-Sepharose column 4 fold. A detailed description of the procedure followed is given in the legend to Figure 8. The activity of the fractions eluting from the Sephadex column was not determined but the protein elution profile was monitored until the protein breakthrough peak had eluted. The following fractions were applied directly to the Blue Dextran-Sepharose affinity column minimizing the time the γ subunit was in dilute solution. This also enabled the preparation

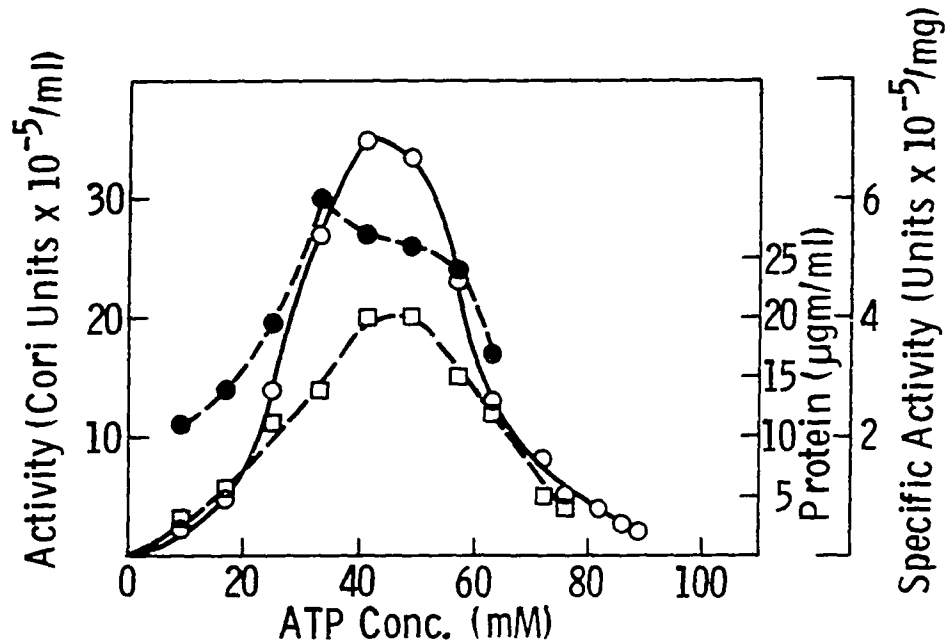
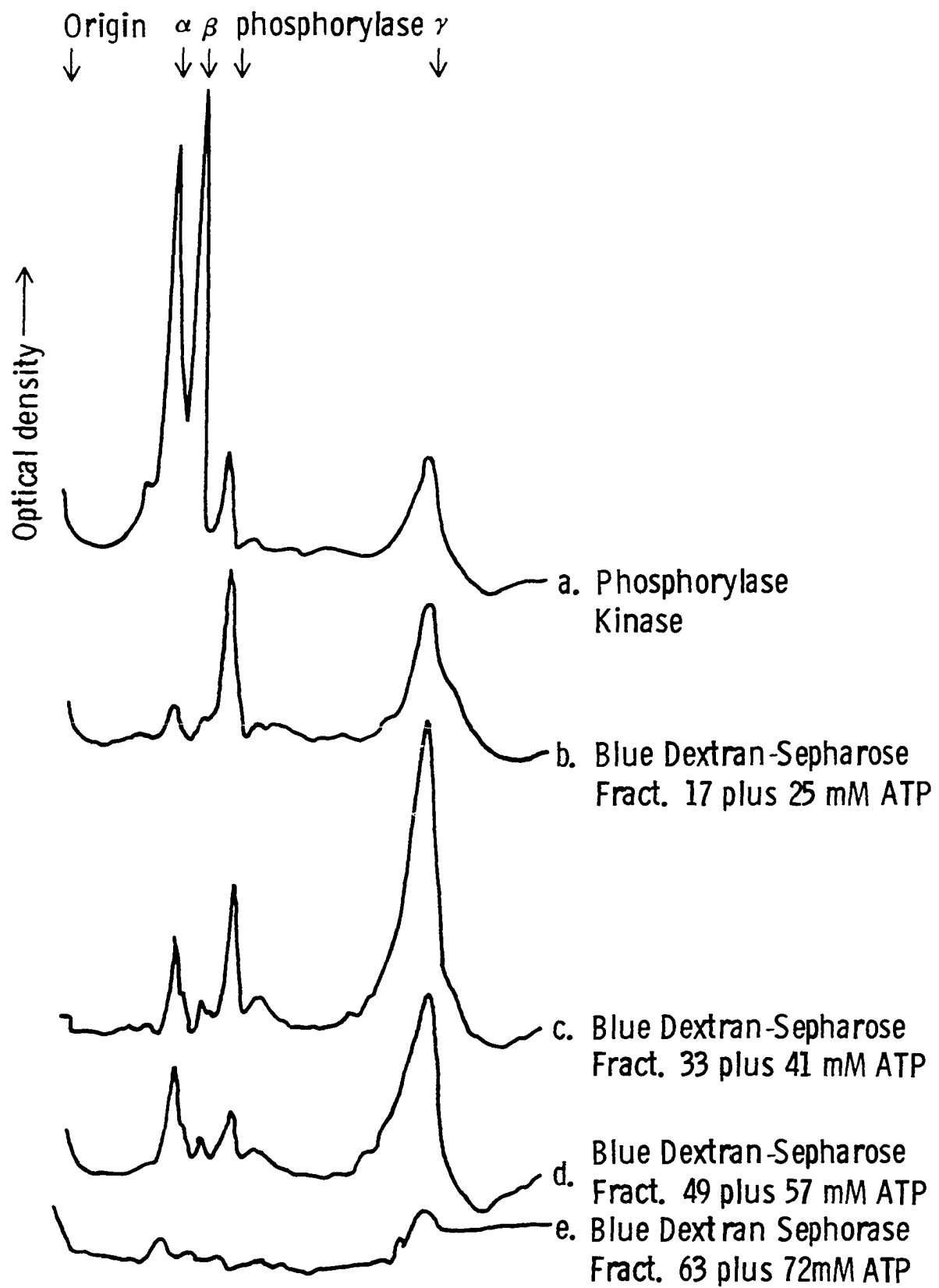


Figure 6. Affinity chromatography of partially purified catalytic subunit. Fractions 23 through 32 (less 29 and 30) eluted from the Sephadex column depicted in Figure 4 were applied to a 2 ml Blue Dextran-Sepharose column. The column was equilibrated and eluted as described in the legend to Figure 3 with a 10 ml linear ATP gradient. The enzyme activity (○), protein concentration (□) and calculated specific activity (●) of the eluted fractions are plotted against the ATP concentration eluting the fraction.

Figure 7. Densitometric tracings of SDS acrylamide gels showing purification of the γ subunit on Blue Dextran Sepharose after Sephadex chromatography. The LiBr incubation conditions are described in the legend to Figure 3, the Sephadex chromatographic procedures are described in the legend to Figure 4 and the Blue Dextran-Sepharose chromatographic procedures are described in the legend to Figure 6. Fractions eluted from the Blue Dextran-Sepharose column depicted in Figure 6 were combined as indicated, denatured, concentrated and electrophoresed as described in Materials and Methods. Tracing 7a is of a gel of native phosphorylase kinase and is also shown in Figure 5g.



of the catalytic subunit to be completed in one long day after the overnight incubation period with LiBr. From 18.8 mg of native phosphorylase kinase (2.35 mg of undissociated γ subunit), 0.9 mg of the catalytic subunit with a specific activity greater than 600,000 was recovered from the affinity column. A partial protein elution profile of the Sephadex column, the elution profile of the affinity column, gels of selected fractions and the molar ratios of contaminating proteins to the catalytic subunit are shown in Figures 8 and 9 and Table 3.

Fractions eluting from 46 mM ATP to 84.5 mM ATP inclusive were pooled and represent the final product. A gel of the product is also shown in Figure 9 and the molar ratio data given in Table 3. The use of two different chromatographic techniques to purify the catalytic subunit of phosphorylase kinase which had been dissociated by incubation with LiBr produces near pure preparations of the γ subunit with recovery of approximately 35% of the γ subunit contained in the incubation mixture. There is no β subunit present in the preparation of the catalytic subunit and only a small amount of α subunit contaminating the preparation. The molar ratio of the γ subunit to the α subunit in the product is 50:1 (Table 3). Phosphorylase is also a minor contaminant in the preparation.

The possibility of limited proteolysis of phosphorylase kinase occurring during the preparation of the catalytic subunit was considered but no evidence was found to suggest it had taken place. No irregularities in the SDS disc gel pattern of phosphorylase kinase were apparent after the enzyme had been incubated with LiBr. Included in Figure 9 are heavily loaded gels of native phosphorylase kinase

Figure 8. Elution profiles determined during the large scale preparation of the catalytic subunit for Sephadex and Blue Dextran-Sepharose chromatography. Inactive phosphorylase kinase was incubated with LiBr for 12 hours at 0° in a mixture containing kinase at 1.4 mg/ml, 5.9% sucrose, 1.2 mM EDTA, 1.2 mM CaCl₂, 29 mM β-glycerophosphate, 88 mM MES and 1.8 M LiBr, pH 7.0. 13.6 ml of incubation mixture was applied to a G-150 Sephadex column (2.5 x 65 cm) equilibrated with 100 mM MES, 0.5 mM CaCl₂, 30 mM 2-mercaptoethanol, pH 7.0. 4.6 ml fractions were collected and the flow rate was 0.6 ml/min. The protein concentration of the early fractions was determined and is shown in the top panel. Fractions 39 through 50 were applied directly to a 9 ml Blue Dextran-Sepharose affinity column equilibrated with the MES buffer. The affinity column elution profile is shown in the bottom panel. The column was eluted with a 40 ml linear ATP gradient collecting 1 ml fractions. The gradient was formed during the elution by mixing 20 mls of 100 mM MES, 0.5 mM CaCl₂, 30 mM 2-mercaptoethanol, pH 7.0 with approximately 20 mls of the same buffer containing 200 mM ATP, pH 7.0 in a gradient forming device. The protein concentration (□) kinase activity at pH 8.2 (○) after diluting 300 fold in dilution buffer are plotted against the ATP concentration of the eluted fraction.

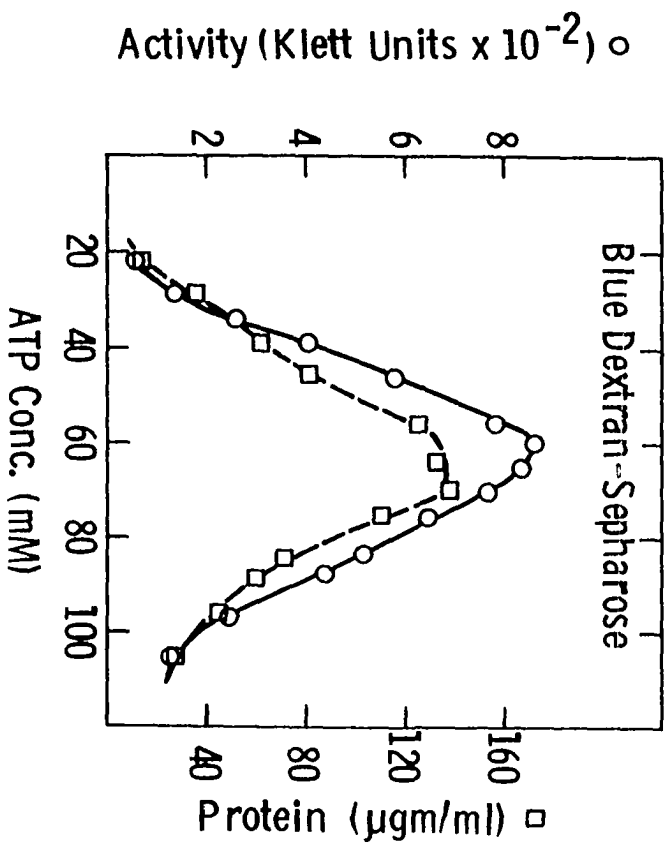
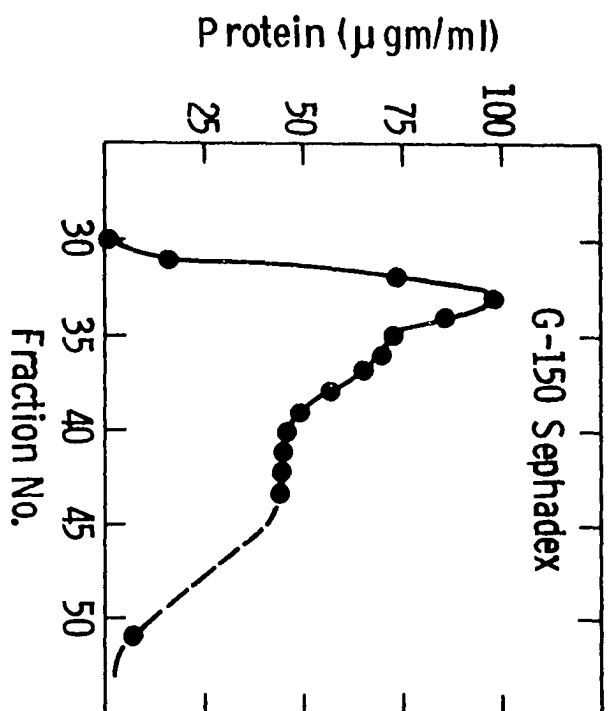


Figure 9. SDS acrylamide gels identifying the γ subunit as the catalytic subunit of phosphorylase kinase. Gel a. is of native phosphorylase kinase. Gel b. is of phosphorylase kinase that had been incubated with 1.8 M LiBr for 3 days using the conditions described in the legend to Figure 8. Gels c. through h. are of fractions eluted from the Blue Dextran-Sepharose column depicted in Figure 8. Gel i. represents the product of the preparation. The fraction at which the sample eluted is c., 34 mM ATP; d., 53 mM ATP; e., 60 mM ATP; f., 70 mM ATP; g., 81 mM ATP; h., 92 mM ATP. Gel i. represents the product of the preparation after pooling fractions 46 mM to 84.5 mM ATP inclusive.

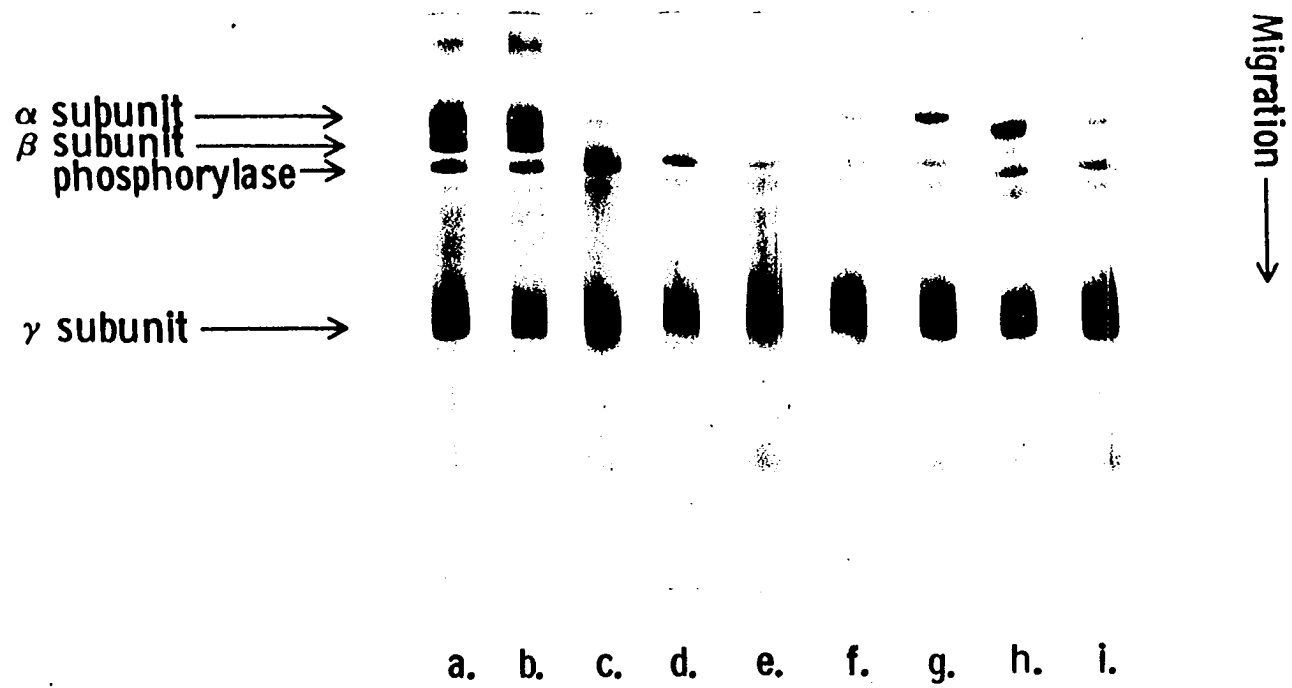


Table 3. Molar ratios of α subunit, β subunit and phosphorylase to γ subunit.^a

Fract. No.	α	β	phos.	γ
34 mM ATP	0.01	0.00	0.27	1.0
53 mM ATP	0.02	0.00	0.09	1.0
60 mM ATP	0.02	0.00	0.03	1.0
70 mM ATP	0.02	0.00	0.02	1.0
81 mM ATP	0.04	0.00	0.03	1.0
92 mM ATP	0.10	0.02	0.06	1.0
Pooled	0.02	0.00	0.04	1.0

^aThe molar ratios were calculated by integration of the gel densities of the SDS acrylamide gels depicted in Figure 9 using the procedures described in the text.

and phosphorylase kinase that had been incubated with 1.8 M LiBr for 3 days. Comparison of the two gels reveal no change in the subunit structure of the enzyme after prolonged incubation.

Stability and storage of the catalytic subunit

Preparations of the purified catalytic subunit were somewhat unstable when stored at 4° and contained high concentrations of ATP in the buffer. The protein concentration of the pooled product was approximately 0.1 mg per ml depending on the size of the preparation. These problems were alleviated by dialyzing the product overnight against 50% glycerol, 100 mM MES, 0.1 mM CaCl₂, 30 mM 2-mercaptoethanol, pH 7.0 and storing the enzyme at -15°. Dialysis also concentrated the enzyme approximately 2 fold which probably aids in the stabilization. Although there was an initial loss of approximately 15% in specific activity during the dialysis, the stored product was found to be completely stable for up to 8 weeks. A less satisfactory method used for some preparations was concentrating the sample by vacuum dialysis against 50 mM ATP, 100 mM MES, 0.1 mM CaCl₂, 30 mM 2-mercaptoethanol, pH 7.0. The specific activity remained high but a large loss of protein occurred when vacuum dialysis was used for concentration. Samples concentrated by both methods were used in the following experiments.

Instability of the catalytic subunit was encountered when diluting the enzyme for assay. Inclusion of phosphorylase b at 1 mg per ml in the 40 mM β -glycerophosphate, 30 mM 2-mercaptoethanol, pH 6.8 dilution buffer helped stabilize the dilute kinase solutions. Plastic test tubes were used throughout the preparation of the catalytic subunit.

Molecular weight of the catalytic subunit

The chromatographic homogeneity and apparent molecular weight of the catalytic subunit was determined by gel filtration as shown in Figure 10. The enzyme chromatographs essentially as one species with an apparent molecular weight of 86,000. The molecular weight of the monomer γ subunit is estimated to be 41,000 to 45,000 by disc gel electrophoresis in the presence of SDS (9, 10) indicating the catalytic subunit as isolated is a dimer.

Effect of Ca^{2+} , EGTA and pH on the activity of purified catalytic subunits

The response of the purified catalytic subunit as well as inactive kinase that had been simply incubated with 1.8 M LiBr to Ca^{2+} , EGTA and pH are in marked contrast to that found for inactive phosphorylase kinase. Figure 11 shows the activity progress curves for the purified catalytic subunit at pH 6.8 and pH 8.2. The typical lag found for the native enzyme is absent and the progress curves are completely linear at both pH 6.8 and pH 8.2.

The requirement of Ca^{2+} for activity by the catalytic subunit was examined using EGTA to chelate Ca^{2+} endogenous to the buffers and enzyme solutions. When 0.1 mM CaCl_2 or 0.1 mM CaCl_2 plus 0.1 mM EGTA was included in the reaction mixture the rate of the reaction was the same as when neither was added. When the activity progress curve was followed with 0.1 mM EGTA in the reaction mixture (Figure 11), a biphasic response was observed. The initial rate appeared to be the same as that seen when EGTA was omitted. The rate rapidly

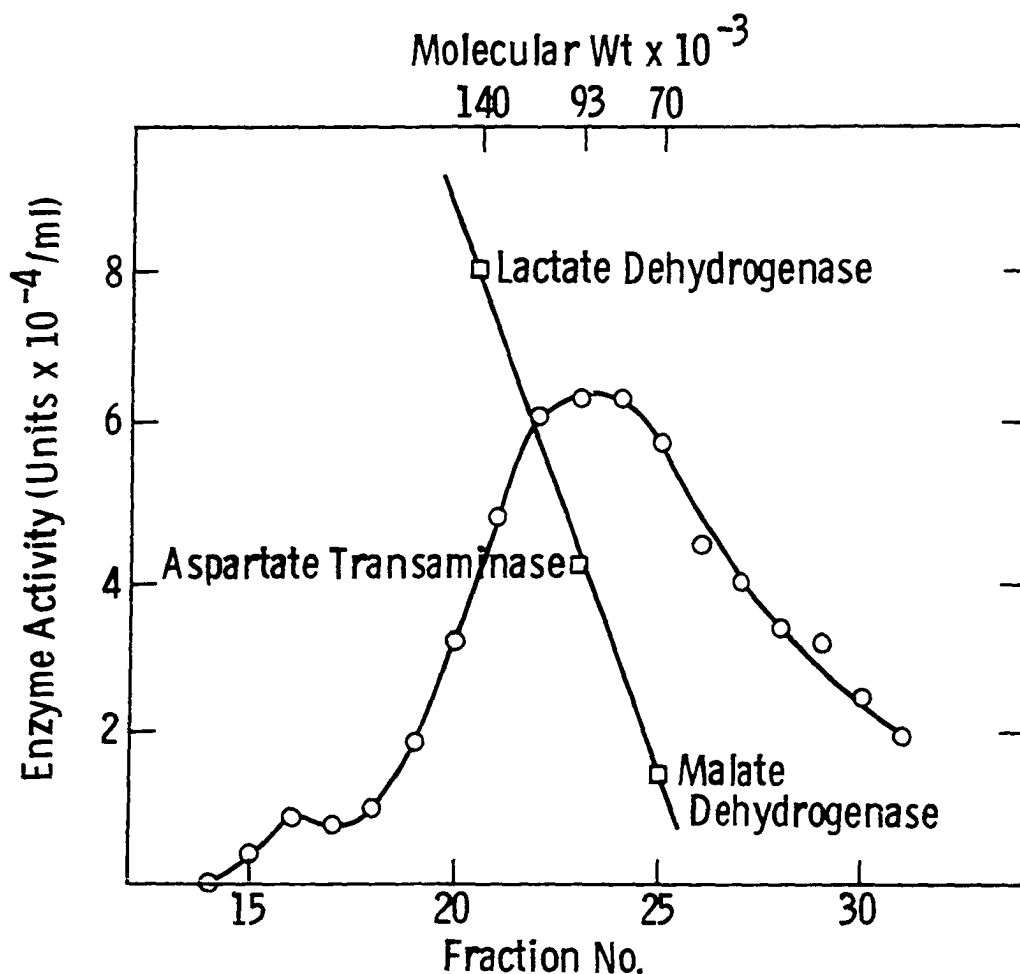


Figure 10. Molecular weight determination of the purified catalytic subunit. 0.4 ml of the catalytic subunit (0.1 mg/ml) was applied to a G-150 Sephadex column (1.15 x 24 cm) equilibrated with 100 mM MES, 0.1 mM CaCl_2 , 30 mM 2-mercaptoethanol, pH 7.0. 0.65 ml fractions were collected and assayed for kinase activity (○). The enzymes LDH, AAT and MDH were used to calibrate the column and assayed as described in materials and methods. Their activity peaks are indicated in the figure (□).

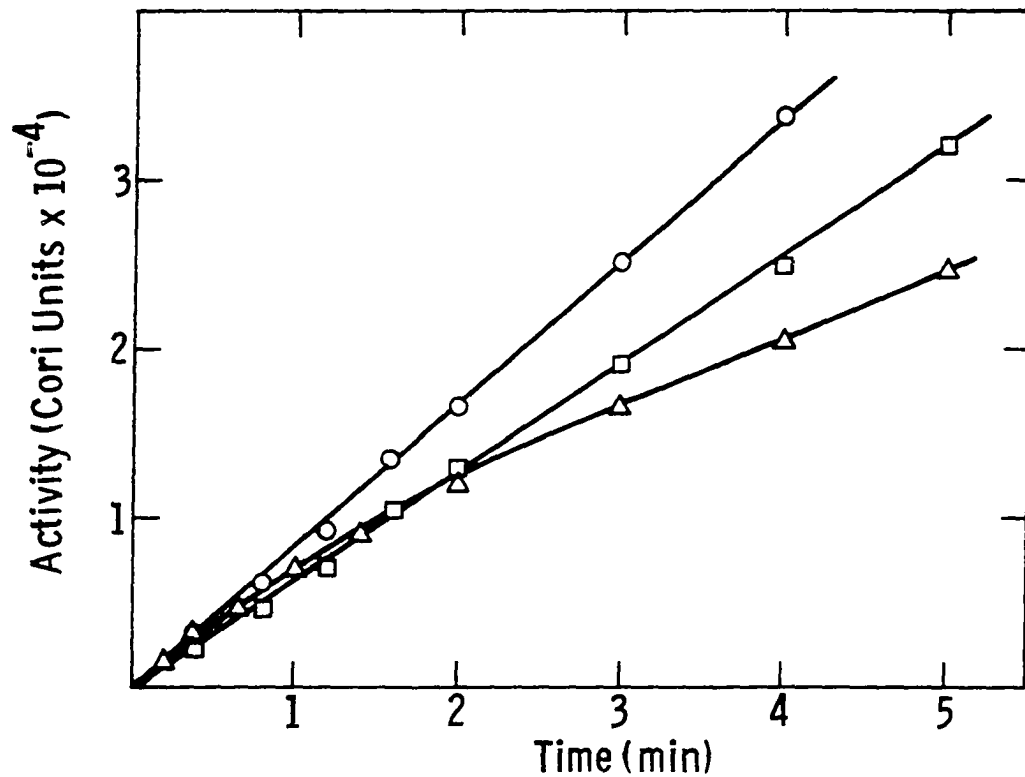


Figure 11. Activity progress curves for the purified catalytic subunit at pH 6.8, pH 8.2 and pH 8.2 in the presence of EGTA. Kinase activity was assayed essentially as described in Materials and Methods except no CaCl_2 was added to the reaction mixture and 0.1 mM EGTA was added where indicated. The reactions were initiated by the addition of purified catalytic subunit (34 μg final) and at various times 50 μl aliquots of reaction mixture were diluted 25 fold and assayed for phosphorylase a activity. Kinase activity at pH 6.8 (\square), at pH 8.2 (\circ), and at pH 8.2 in the presence of 0.1 mM EGTA (\triangle).

decreases and after about 2 minutes stabilizes at a rate approximately 40% of the initial rate. The progress curve for the reaction is linear after the initial loss of activity. The effect of incubating the purified catalytic subunit with Ca^{2+} and EGTA on its activity is shown in Figure 12. When the catalytic subunit is incubated with 0.1 mM Ca^{2+} , diluted and assayed in the presence of 0.1 mM Ca^{2+} , the reaction progress curve is linear. When the same Ca^{2+} incubated enzyme is assayed in the presence of 0.1 mM EGTA, a time dependent loss of activity is seen during the reaction. The effect of EGTA on the kinase reaction after incubation with Ca^{2+} is the same as when the incubation was not done. However, when the catalytic subunit was incubated with 0.1 mM EGTA, diluted and assayed in the presence of 0.1 mM Ca^{2+} or 0.1 mM EGTA, the activity progress curves are identical. The progress curves are linear and at the rate seen for the Ca^{2+} incubated enzyme which was assayed in the presence of EGTA after the transition to a reduced rate. Additionally, the loss of activity caused by incubating the enzyme with EGTA was not reversed by including Ca^{2+} in the reaction mixture.

The activity pH profile curve of the catalytic subunit is shown in Figure 13. Unlike inactive phosphorylase kinase which has almost no activity at pH 6.8, the catalytic subunit has near full activity at neutral pH and the pH 6.8/8.2 activity ratio is approximately 0.70. The pH optimum is pH 8.2 for the purified catalytic subunit as well as all other forms of phosphorylase kinase.

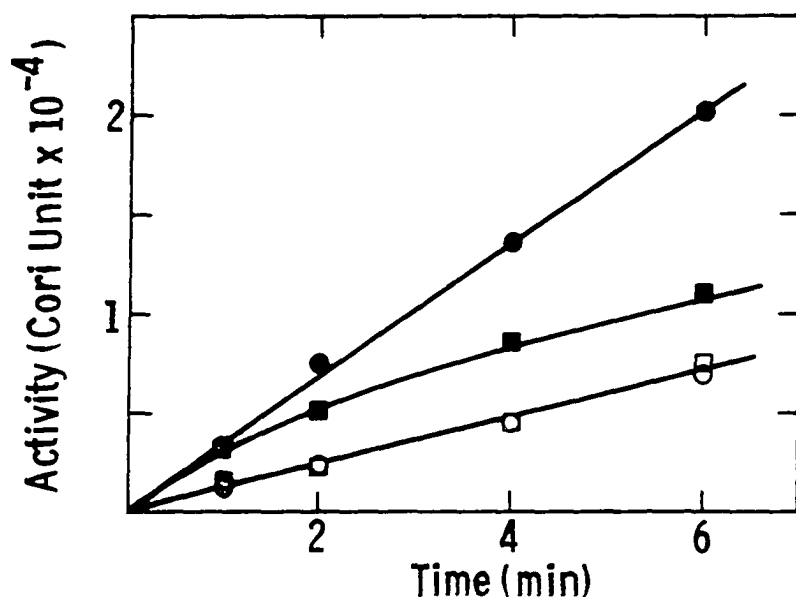


Figure 12. Effect of incubation with Ca^{2+} and EGTA on the activity of the purified catalytic subunit. The purified catalytic subunit ($4 \mu\text{g}/\text{ml}$ in incubation mixture) was incubated with phosphorylase *b* at $10 \text{ mg}/\text{ml}$, 42 mM Tris, 55 mM β -glycerophosphate, 15 mM 2-mercaptoethanol, 3 mM ATP, 1.5 mM MES, 0.76% glycerol, and 0.1 mM CaCl_2 or 0.1 mM EGTA, pH 8.1 for 5 minutes at 30° . The mixture was diluted 21 fold in 40 mM β -glycerophosphate, 30 mM 2-mercaptoethanol, pH 6.8 and assayed for kinase activity in the presence of 0.1 mM CaCl_2 or 0.1 mM EGTA as described in the legend to Figure 11. Kinase activity after incubation with CaCl_2 and assayed in the presence of Ca^{2+} (●) or EGTA (■). Activity after incubation with EGTA and assayed in the presence of Ca^{2+} (○) or EGTA (□).

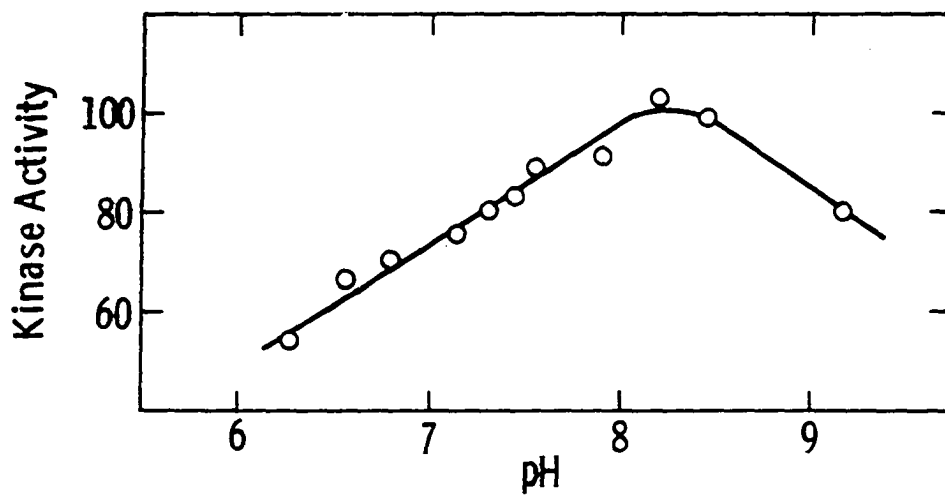


Figure 13. The activity-pH profile for the purified catalytic subunit. The kinase activity was determined essentially as described in Materials and Methods except no CaCl_2 was added to the reaction mixtures. The reaction was initiated by the addition of purified catalytic subunit (42 $\mu\text{g}/\text{ml}$ final) and after 5 minutes reaction time, 50 μl aliquots were diluted 25 fold and assayed for phosphorylase a activity. The pH of the individual reaction mixtures was determined using a pH meter.

DISCUSSION

Phosphorylase kinase from rabbit muscle is a structurally complicated molecule composed of three different polypeptide chains. The function of none of these subunits has been established with certainty prior to this report. The enzyme is highly regulated by several distinct mechanisms and progress on understanding them have been hampered by the inherent complexity of the molecule. The isolation of the catalytic subunit allows new approaches to be taken in studying the mechanisms of action of phosphorylase kinase and inferences to be made concerning the regulation of the holoenzyme.

Relatively simple procedures are used in the purification scheme for preparing the catalytic subunit. The purified γ subunit is completely devoid of the β subunit and contaminated by only a small amount of the α subunit. The yield of γ subunit protein and activity is 35 to 40% of that contained in the LiBr incubation mixture.

The use of concentrated salt solutions to disrupt the noncovalent structure of proteins has been described for several systems and salts such as LiBr tend to solubilize, dissociate, and denature proteins (42). The effect of LiBr on phosphorylase kinase is likely to be all three. In preparing the LiBr incubation mixture care was taken to insure that the solutions were near 0° and to add the LiBr last in small amounts with thorough mixing to avoid locally high concentrations. If care was not taken or the LiBr concentration was too high, inactivation occurred which was almost certainly due to denaturation of the enzyme. Incubation of inactive phosphorylase

kinase with 1.8 M LiBr overnight was found to effectively dissociate the enzyme. Since the specific activity of the LiBr dissociated phosphorylase kinase was usually about 95,000 units per mg, the specific activity of the purified catalytic subunit would be predicted to be $8 \times 95,000$ or 760,000 units per mg, since $1/8$ of the total protein of phosphorylase kinase is γ subunit. This high specific activity was approached in peak fractions eluted from the Blue Dextran-Sepharose during the purification of the catalytic subunit and fractions having specific activities greater than 650,000 units per mg were isolated.

Incubation of phosphorylase kinase with LiBr caused time dependent changes in the activity and state of dissociation of phosphorylase kinase. Large fluctuations in the pH 8.2 activity, a steady increase in the pH 6.8 activity and decrease in sensitivity to EGTA inhibition were observed during the incubation period. Sucrose density centrifugation of phosphorylase kinase that had been incubated only 2 hours with 1 M LiBr caused dissociation of native phosphorylase kinase to several catalytically active molecular weight forms having S values from 13S to 8S and 5S. The activity elution profile from Sephadex columns of inactive kinase that had been dissociated with 1.8 M LiBr also indicated complete dissociation had not occurred. The evidence would suggest that incubation of kinase with LiBr dissociates the enzyme through several stages before arriving at the dimeric catalytic subunit. It is likely that during the process varying amounts of α subunit and β subunit are lost from the γ subunit with some forms being less active than others. This would explain the fluctuations in pH 8.2 activity

observed during the dissociation process. If this suggestion is correct, species of phosphorylase kinase with subunit structures intermediate to the $\alpha_4\beta_4\gamma_4$ native enzyme and γ_2 catalytic subunit could possibly be isolated for study. Isolation and study of enzyme complexes such as $(\alpha\gamma)$ or $(\beta\gamma)$ or even $(\alpha_1\beta_1\gamma_1)$ may provide valuable information about the regulatory processes of phosphorylase kinase.

Possible approaches for isolating a phosphorylase kinase of intermediate molecular weight would be to decrease the concentration of the LiBr in the incubation mixture or time of incubation. The use of ATP as a dissociating agent may prove advantageous in isolating intermediate molecular weight forms of phosphorylase kinase. As indicated in the results, 8S and 14S species were found for kinase dissociated by incubating with 100 M ATP. The 8S and 14S forms of ATP dissociated phosphorylase kinase had completely lost its sensitivity to EGTA. The complete loss of EGTA inhibition of kinase activity may be an indication of dissociation to intermediate molecular weight forms. Total lack of EGTA inhibition was also found for phosphorylase kinase that had been incubated with 0.83 M LiBr.

Sephadex chromatography of LiBr dissociated phosphorylase kinase is essential in the purification of the catalytic subunit. The value of gel filtration in the purification scheme can be seen by comparing the specific activities of fractions eluted from the Blue Dextran-Sephadex columns when both Sephadex and Blue Dextran-Sephadex chromatography were used to those when Sephadex chromatography was omitted. The specific activities of the peak fractions eluted from

the affinity resin were nearly doubled by partially purifying the LiBr treated kinase on Sephadex before applying the dissociated enzyme to the Blue Dextran-Sepharose column.

The purification of the γ subunit from the β subunit was facilitated by an interaction between the β subunit and the gel matrices of Sephadex and Blue Dextran-Sepharose. No visible turbidity or precipitation was seen in the LiBr incubation mixtures. Chromatography of LiBr treated kinase on either gel resulted in the near complete disappearance of the β subunit from the eluted fractions. The β subunit may strongly bind to the polysaccharide chromatographic materials preventing its elution. Formation of glycogen-phosphorylase kinase complexes are known to occur (30). Another explanation is that the β subunit precipitates in the gel matrices when the LiBr is removed from the protein solution during chromatography. Molecular sieve chromatography of LiBr dissociated phosphorylase kinase could result in the recovery of the β subunit if the chromatographic procedures were modified. Either substitution of a nonpolysaccharide chromatographic material for Sephadex or inclusion of LiBr in the elution buffer, depending upon the nature of the interaction of the β subunit and the gel, should result in normal chromatography of the β subunit.

Blue Dextran-Sepharose was used as an affinity resin for the purification of the catalytic subunit of phosphorylase kinase. The interaction between the Blue Dextran ligand and the catalytic subunit was not simply ionic in that 1 M NaCl was ineffective as an eluting agent. The elution properties of the catalytic subunit from Blue

Dextran-Sepharose were unusual and differed from that found for other kinases and dehydrogenases. For example, the catalytic subunit of cyclic-AMP dependent protein kinase is eluted from the resin by 1 mM ATP/5 mM Mg^{2+} or 0.2 M KCl (43). The phosphorylase kinase substrate MgATP was ineffective in eluting the γ subunit of phosphorylase kinase from the affinity column. Extremely high concentrations of ATP or ADP were required for elution with the peak activity fractions eluting near 50 mM nucleotide. Additionally, the activity of the native enzyme was not affected during the ATP incubation experiments unless the concentration of ATP was greater than 25 mM. These data would suggest that the catalytic subunit is eluted from Blue Dextran-Sepharose by conformation changes in enzyme structure induced by ATP and not a simple displacement of the enzyme from the Blue Dextran ligand by ATP. LiBr was also found to be an effective eluting agent at concentrations (0.65 M) near that found to promote dissociation of the native enzyme. Although the forces involved in binding and eluting the catalytic subunit from Blue Dextran-Sepharose are not certain and may involve binding of the enzyme at the catalytic site or other site(s), the affinity gel was found to be extremely effective in the purification of the catalytic subunit of phosphorylase kinase.

The capacity of Blue Dextran-Sepharose was approximately 1 mg of phosphorylase kinase dissociated by LiBr per ml of resin. The capacity of the gel for LiBr dissociated phosphorylase kinase that had been partially purified by Sephadex chromatography was not determined. Using the purification scheme, 1 ml of resin was found adequate for a preparation beginning with at least 3 mg of phosphorylase kinase.

The protein concentration dependence of LiBr dissociation of phosphorylase kinase was tested over a narrow range (0.75 mg/ml to 1.4 mg/ml) and no effect was seen. Larger preparations of the catalytic subunit would require either larger gel filtration columns or more concentrated protein solution in the LiBr incubation mixture. Increasing the concentration of phosphorylase kinase in the incubation mixture would likely be the better choice.

The characteristically low pH 6.8 activity of nonactivated kinase is lost during the preparation of the catalytic subunit. During the incubation of kinase with LiBr, the enzyme dissociates and the pH 6.8 activity increases from less than 5% to approximately 70% of the pH 8.2 activity. An increase in the pH 6.8 activity of phosphorylase kinase also occurs when the enzyme becomes activated by phosphorylation or trypsinization. Cyclic-AMP dependent protein kinase activated phosphorylase kinase has a pH 6.8/8.2 activity ratio of 0.38. Cohen (9) correlated the phosphorylation of a specific seryl residue in the β subunit with the increase in the pH 6.8 activity. He also reported that phosphorylation of a specific seryl residue in the α subunit was not associated with any change in activity of phosphorylase kinase but was important in regulating the dephosphorylation of the β subunit. The pH 6.8/8.2 activity ratio of autoactivated phosphorylase kinase is 0.5. Wang, et al. (15) found the increase in the pH 6.8 activity occurring during autophosphorylation correlated with total incorporation of phosphate into both the α subunit and β subunit. Trypsin activated phosphorylase kinase has a pH 6.8/8.2 activity ratio of 0.67 and the increase in the pH 6.8 activity during trypsinization of the kinase

seems to parallel the degradation of the α subunit. The pH 6.8/8.2 activity ratio of the purified catalytic subunit of phosphorylase kinase is 0.7. The increase in pH 6.8 activity and in the pH 6.8/8.2 activity ratio occurs during dissociation of the holoenzyme by LiBr and remains constant thereafter. This evidence suggests that the pH 6.8 activity of nonactivated phosphorylase kinase is strongly suppressed by a regulatory subunit but inactive kinase possesses full pH 6.8 catalytic potential. Protein kinase phosphorylation partially relieves the inhibition of pH 6.8 activity, more complete phosphorylation during the auto-activation reaction results in less inhibition and trypsinization results in full pH 6.8 activity being expressed. Whether the α subunit or β subunit or both inhibit the pH 6.8 activity is not known although all three possibilities have been suggested. Characterization of the properties of ($\alpha\gamma$) or ($\beta\gamma$) hybrid forms should provide insight into the regulation of the pH 6.8 activity of phosphorylase kinase.

Another interesting aspect of the pH dependence of phosphorylase kinase is the pH activity profile found when the TN-I component of troponin is used as the substrate for the kinase reaction. Both non-activated and protein kinase activated phosphorylase kinase have the same pH 6.8/8.2 activity ratios of 0.7 when assayed in this system indicating full expression of the pH 6.8 activity of the enzyme. An investigation into the substrate dependence of the pH 6.8 activity may provide useful information about regulation of kinase activity. This would become particularly significant if troponin is found to be a physiological substrate for phosphorylase kinase.

The absolute requirement of Ca^{2+} for activity of all phosphorylase kinase forms previously described is fundamental to the in vivo regulation of glycogenolysis. The purified catalytic subunit of phosphorylase kinase appears not to have an absolute requirement of Ca^{2+} for catalytic activity. The Ca^{2+} chelating reagent EGTA was used in studying the effects of the metal on the activities of nonactivated kinase and the purified catalytic subunit. When nonactivated phosphorylase kinase was assayed in the presence of EGTA, approximately 97% inhibition of the pH 8.2 activity was observed. However, the effect of EGTA on the purified catalytic subunit indicated a complex role(s) for Ca^{2+} . The addition of 0.1 M EGTA to the assay system causes a time dependent reduction in the activity of the purified catalytic subunit. The initial velocity in the presence of EGTA is the same as that found for the catalytic subunit when assayed in the absence of EGTA. The reaction velocity rapidly decays over a 2 minute period and stabilizes at approximately 40% of the initial velocity. The reaction progress curves were linear after the initial loss of activity indicating the effect was not due to denaturation of the enzyme in the reaction mixture containing EGTA. It is likely that the effect of EGTA is in binding calcium and not an EGTA-protein interaction. The addition of equal molar amounts of Ca^{2+} and EGTA to the reaction mixture resulted in the same activity as when neither was added. The partial loss of activity caused by the addition of EGTA to the reaction mixtures indicates that the purified catalytic subunit does bind Ca^{2+} and the chelation of Ca^{2+} in the assay system affects the enzyme's activity. A possible

explanation for the EGTA effect is that the catalytic subunit binds the metal so tightly that it is not completely removed from the enzyme by EGTA. A more likely explanation is that the purified catalytic subunit does not have an absolute Ca^{2+} requirement for activity but Ca^{2+} stabilizes the enzyme in an active conformation. The removal of Ca^{2+} from the enzyme could cause a time dependent transition in structure to a less active conformation. This concept is supported by experiments showing the effect of incubating the purified catalytic subunit with Ca^{2+} and EGTA on its activity. The response of the catalytic subunit to the presence of Ca^{2+} or EGTA in the assay mixture after incubation with Ca^{2+} was the same as when the incubation was not done. When the enzyme was assayed in the presence of EGTA and Ca^{2+} after incubation with EGTA, the activity progress curve was completely linear and at a reduced rate. This suggests that a transition to a less active form occurred during the incubation with EGTA which was not reversed by the presence of Ca^{2+} in the reaction mixture.

The interpretations of the experimental results on the effect of Ca^{2+} and EGTA on the activity of the purified catalytic subunit imply that another subunit regulates native phosphorylase kinase's activity in response to Ca^{2+} . In the absence of Ca^{2+} the α subunit or β subunit or both may completely inhibit the activity of the native enzyme. The inhibition is relieved by the binding of Ca^{2+} by the enzyme. An alternative explanation could be that the catalytic

subunit of the native enzyme possesses a requirement of Ca^{2+} for activity but the metal requirement is somehow lost during the LiBr treatment.

The regulation of enzyme activity by small compounds binding to regulatory subunits are known for other systems. An interesting example is the regulation of cyclic-AMP dependent protein kinase by cyclic-AMP (44). The regulatory and catalytic subunits are associated to form an inactive holoenzyme complex. The binding of cyclic-AMP by the regulatory subunit results in dissociation of the holoenzyme with expression of catalytic activity. The previous discussion about possible mechanisms of regulation of phosphorylase kinase by Ca^{2+} suggest that the two kinases may be regulated by similar mechanisms. The analogy is that the binding of Ca^{2+} and cyclic-AMP by the regulatory subunit of the enzymes results in the loss of inhibition by the regulatory subunit. In the cyclic-AMP dependent protein kinase example, cyclic-AMP causes a conformation change upon binding that promotes dissociation and results in expression of activity. Phosphorylase kinase could be similar in that the binding of Ca^{2+} by a regulatory subunit also promotes a conformation change allowing for the expression of activity without dissociation occurring.

Other similarities exist between the two enzymes. The molecular weights of the catalytic subunits of the two proteins are similar; 41,300 for cyclic-AMP dependent protein kinase (45) and approximately 43,000 for phosphorylase kinase. The amino acid compositions of the catalytic subunits are also similar (Table 4). Two exceptions

Table 4. The amino acid compositions of the catalytic subunits of phosphorylase kinase and cyclic-AMP dependent protein kinase.

Amino Acid	γ Subunit of phosphorylase kinase		Catalytic subunit of cyclic- AMP dependent protein kinase
	mol/45,000 gm ^a	mol/42,000 gm ^b	mol/41,000 gm ^c
Lysine	23	24	34
Histidine	10	9	7
Arginine	25	24	16
Aspartic acid	37	33	35
Threonine	20	18	13
Serine	21	20	17
Glutamic	45	43	44
Proline	11	19	15
Glycine	24	22	24
Alanine	23	22	24
Valine	28	25	20
Methionine	10	10	5
Isoleucine	19	17	19
Leucine	37	36	32
Tyrosine	18	15	14
Phenylalanine	19	15	24
Tryptophan	4	--	9
Cysteine	8	--	3
Total Residues	390	352	355

^aComposition as reported by Cohen (9).

^bComposition as reported by Hayakawa, et al. (11).

^cComposition as reported by Bechtel, et al. (45).

are lysine and phenylalanine which are less abundant in the γ subunit. All other amino acids are found to be near equally or more abundant in the larger γ subunit of phosphorylase kinase. An interesting area of research would be to determine if homologies in the protein structures of the two protein kinases exist.

The number of proteins that will serve as substrates for phosphorylase kinase is very limited. Its physiological substrate, phosphorylase, is the only protein found to be a good substrate. Phosphorylase kinase in the autocatalytic reaction and the TN-I component of troponin will serve as substrates. Casein and the TN-T component of troponin have been reported to be very poor substrates (30, 46). Synthetic peptides representing the N-terminal region of phosphorylase have also been shown to be good substrates for the enzyme. The substrate specificity of the purified catalytic subunit should be carefully examined. The high degree of substrate specificity found for native phosphorylase kinase may be a property of the catalytic subunit. Another possibility is that the regulatory subunits of the enzyme may restrict the number of substrates that phosphorylase kinase will phosphorylate. A choice between these alternatives could be made by fully characterizing the substrate specificity of the purified catalytic subunit.

The report by Fisher et al. (27) comparing the dogfish and rabbit muscle phosphorylase kinases showed that the molecular weight and subunit structure of the enzymes are similar. Some differences in the properties were described and this report indicates that the enzymes

are quite dissimilar. The γ subunit of dogfish phosphorylase kinase was found to be very similar to actin. The amino acid compositions of dogfish actin and the γ subunit of dogfish kinase were nearly identical. The γ subunit was shown to interact with dogfish heavy meromyosin to form typical decorated filaments which is a characteristic of actin. The report included evidence that the catalytic subunit of dogfish kinase is the β subunit. This report establishing that the catalytic subunit of rabbit muscle phosphorylase kinase is the γ subunit suggests that even greater differences exist between phosphorylase kinases isolated from the distantly related vertebrates.

The purified catalytic subunit of rabbit muscle phosphorylase kinase has a specific activity approaching that reported for nonactivated phosphorylase kinase on a molar basis. Activation of native nonactivated kinase results in a 50% to 100% increase in the total pH 8.2 activity. This implies that the total pH 8.2 activity of the native activated enzyme forms is not found in the purified catalytic subunit. A possible explanation for the difference in the pH 8.2 activity of activated phosphorylase kinase and the purified catalytic subunit on a molar basis is that the activity of the γ subunit of the native enzyme can be stimulated by another subunit. Phosphorylation or trypsinization of the native enzyme could cause the α subunit or β subunit to stimulate the catalytic activity of the γ subunit. Another explanation is that phosphorylase kinase has two distinct catalytic subunits. Activation of the native enzyme allows the activity of a catalytic subunit other than the γ subunit to be expressed. Purification and study of the

α and β subunits should resolve this question. Perhaps the β subunit of phosphorylase kinase will be found to be catalytically active as has been reported for the β subunit of the dogfish enzyme.

Studies determining the properties of the purified catalytic subunit of phosphorylase kinase suggest that the α subunit and/or β subunit possess regulatory functions in the native enzyme. The purified catalytic subunit has lost the absolute requirement of calcium for activity indicating the α and/or β subunit completely inhibit the activity of the native enzyme in absence of calcium. The pH 6.8 activity of phosphorylase kinase is strongly inhibited in nonactivated kinase, somewhat repressed in kinase activated by phosphorylation and at full activity for kinase activated by trypsinization as well as for the purified catalytic subunit. The evidence suggests that the catalytic activity of the γ subunit can be inhibited by the α and/or β subunit of the native enzyme. Furthermore, if the arguments based on the specific activities of the native phosphorylase kinases and the purified catalytic subunit are correct, the α and/or β subunit either can stimulate the activity of the γ subunit in the native enzyme or possess phosphorylase kinase activity themselves.

The isolation and study of the catalytic subunit has allowed interesting inferences to be made concerning the regulatory features of phosphorylase kinase. Hopefully this research has established solid groundwork on which more definitive experimentation can be done on concerning mechanisms of regulation. The γ subunit of phosphorylase kinase is now available for study. The results contained in this report

should facilitate the purification of the other subunits of the enzyme. Important observations concerning the mechanisms of regulation and action should result from studies of all three purified subunits as well as recombinant forms. Hopefully, new insights into the enzymology of phosphorylase kinase will be gained using the approach taken in this work.

LITERATURE CITED

1. Green, A. A. and Cori, G. T. (1943), J. Biol. Chem., 151, 21.
2. Fisher, E. H., Graves, D. J., Crittenden, E. R. S. and Krebs, E. G. (1959), J. Biol. Chem., 234, 1698.
3. Meyer, W. L., Fisher, E. H., and Krebs, E. G. (1964), Biochemistry, 3, 1033.
4. Krebs, E. G., Huston, R. B. and Hunkeler, F. L. (1968), Advan. Enzyme Regul., 6, 245.
5. Ozawa, E., Hosoi, K. and Ebashi S. (1967), J. Biochem. (Tokyo), 61, 531.
6. Brostrom, C. O., Hunkeler, F. L. and Krebs, E. G. (1971), J. Biol. Chem., 246, 1961.
7. Krebs, E. G., Graves, D. J. and Fisher, E. H. (1959), J. Biol. Chem., 234, 2867.
8. Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L. and Fisher, E. H. (1964), Biochemistry, 3, 1022.
9. Cohen, P. (1973), Eur. J. Biochem., 34, 1.
10. Hayakawa, T., Perkins, J. P., Walsh, D. A. and Krebs, E. G. (1973), Biochemistry, 12, 567.
11. Hayakawa, T., Perkins, J. P. and Krebs, E. G. (1973), Biochemistry, 12, 574.
12. Jennissen, H. P. and Heilmeyer, L. M. G. (1974), FEBS Lett., 42, 77.
13. Kim, G. and Graves, D. J. (1973), Biochemistry, 12, 2090.
14. Carlson, G. M. and Graves, D. J. (1976), J. Biol. Chem., 251, 7480.
15. Wang, J. H., Stull, J. T., Huang, T-S. and Krebs, E. G. (1976), J. Biol. Chem., 251, 4521.
16. Yeaman, S. J. and Cohen, P. (1974), Biochem. Soc. Trans., 2, 931.
17. Yeaman, S. J. and Cohen, P. (1975), Eur. J. Biochem., 51, 93.
18. Cohen, P. and Antoniow, J. F. (1973), FEBS Lett., 34, 43.

19. Graves, D. J., Hayakawa, T., Horvitz, R. A., Beckman, E. and Krebs, E. G. (1973), Biochemistry, 12, 580.
20. Huston, R. B. and Krebs, E. G. (1968), Biochemistry, 7, 2116.
21. Fisher, E. H. and Krebs, E. G. (1955), J. Biol. Chem., 216, 121.
22. Ozawa, E. and Ebashi, S. (1967), J. Biochem. (Tokyo), 62, 285.
23. Kilimann, M. and Heilmeyer, L. M. G. (1977), Eur. J. Biochem., 73, 191.
24. Tabatabai, L. B. (1976), Ph.D. Thesis, Iowa State University.
25. Stull, J. T., Brostrom, C. O. and Krebs, E. G. (1972), J. Biol. Chem., 247, 5272.
26. Gergely, P., Vereb, G. and Bot, G. (1974), Acta Biochim. et Biophys. Acad. Sci. Hung., 9, 223.
27. Fisher, E. H., Blum, H. E., Byers, B., Heizmann, G. W., Kerrick, P. L., Malencik, D. A. and Pocinwog, S. (1976), in Metabolic Interconversion of Enzymes, 1975, S. Shaltiel, Ed., Springer-Verlag, Berlin Heidelberg.
28. Tessmer, G. W., Skuster, J. R., Tabatabai, L. B. and Graves, D. J. (1977), J. Biol. Chem., (in press).
29. Mayer, S. E. and Krebs, E. G. (1970), J. Biol. Chem., 245, 3153.
30. DeLange, R. J., Kemp, R. G., Riley, W. D., Cooper, D. A. and Krebs, E. G. (1968), J. Biol. Chem., 243, 2200.
31. Fisher, E. H. and Krebs, E. G. (1958), J. Biol. Chem., 231, 65.
32. Illingworth, B. and Cori, G. T. (1953), Biochem. Prep., 3, 1.
33. Kastenschmidt, L. L., Kastenschmidt, J. and Helmreich, E. (1968), Biochemistry, 7, 3590.
34. Bradford, M. M. (1976), Anal. Biochem., 72, 248.
35. Reimann, E. M., Walsh, D. A. and Krebs, E. G. (1971), J. Biol. Chem., 246, 1986.
36. Ryan, L. D. and Vestling, C. S. (1974), Arch. Biochem. Biophys., 160, 279.
37. Worthington Enzyme Manual (1975), Worthington Biochemical Corporation, Freehold, N.J.

38. Furbish, F. S., Fonda, M. L. and Metzler, D. E. (1969), Biochemistry, 8, 5169.
39. Glynn, I. M. and Chappell, J. B. (1964), Biochem. J., 90, 147.
40. Carlson, G. M. and Graves, D. J. (1976), Biochemistry, 15, 4476.
41. Stellwagen, E. (1977), Acc. Chem. Res., 10, 92.
42. Jenks, W. P. (1969), in Catalysis in Chemistry and Enzymology, McGraw-Hill Book Company, New York, N.Y. p. 358.
43. Witt, J. J. and Roskoski, R. (1975), Biochemistry, 14, 4503.
44. Brostrom, M. A., Reimann, D. A. and Krebs, E. G. (1970), Advan. Enzyme Regul, 8, 191.
45. Bechtel, P.J., Beavo, J. A. and Krebs, E. G. (1977), J. Biol. Chem., 252, 2691.
46. Perry, S. V. and Cole, H. A. (1973), Biochem. J., 131, 425.

ACKNOWLEDGMENTS

To Donald J. Graves for the help, encouragement and enthusiasm expressed while I was his student. His personal friendship and scientific professionalism has made our association an enjoyable, exciting and fruitful experience.

To all the members of the research group for their friendship and help.

I especially thank my family, Charlotte, Eileen and Patty for their support and the sacrifices they made during the past few years.